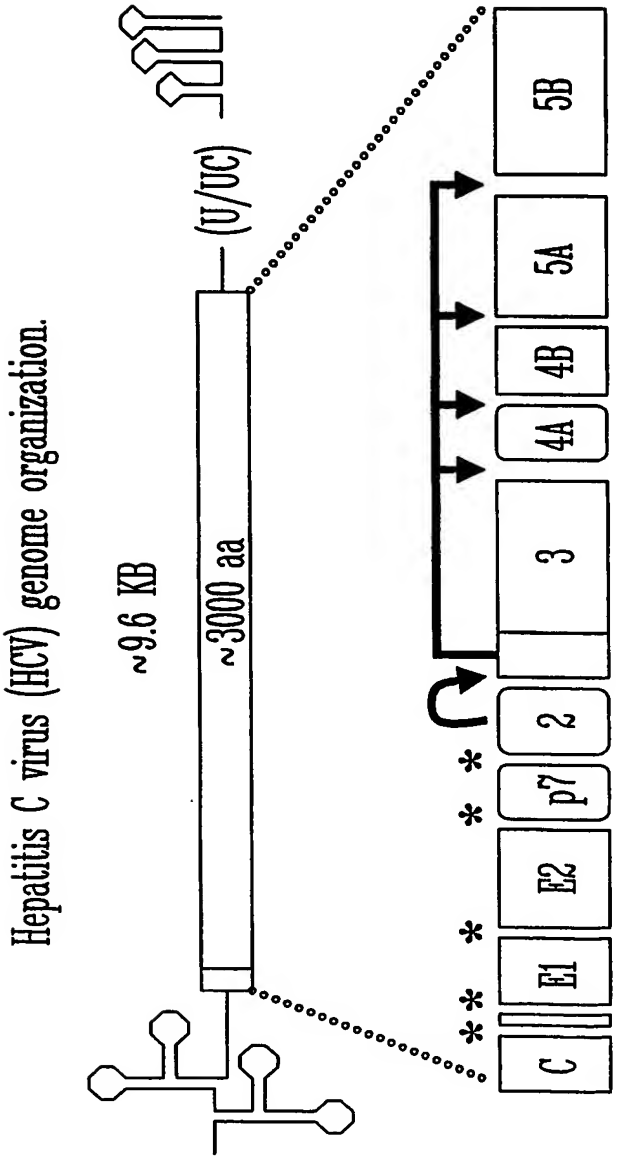

Hepatitis C virus (HCV) genome organization.
~9.6 KB
~3000 aa
(U/UC)
C
E1
E2
p7
2
3
4A
4B
5A
5B

Fig. 1

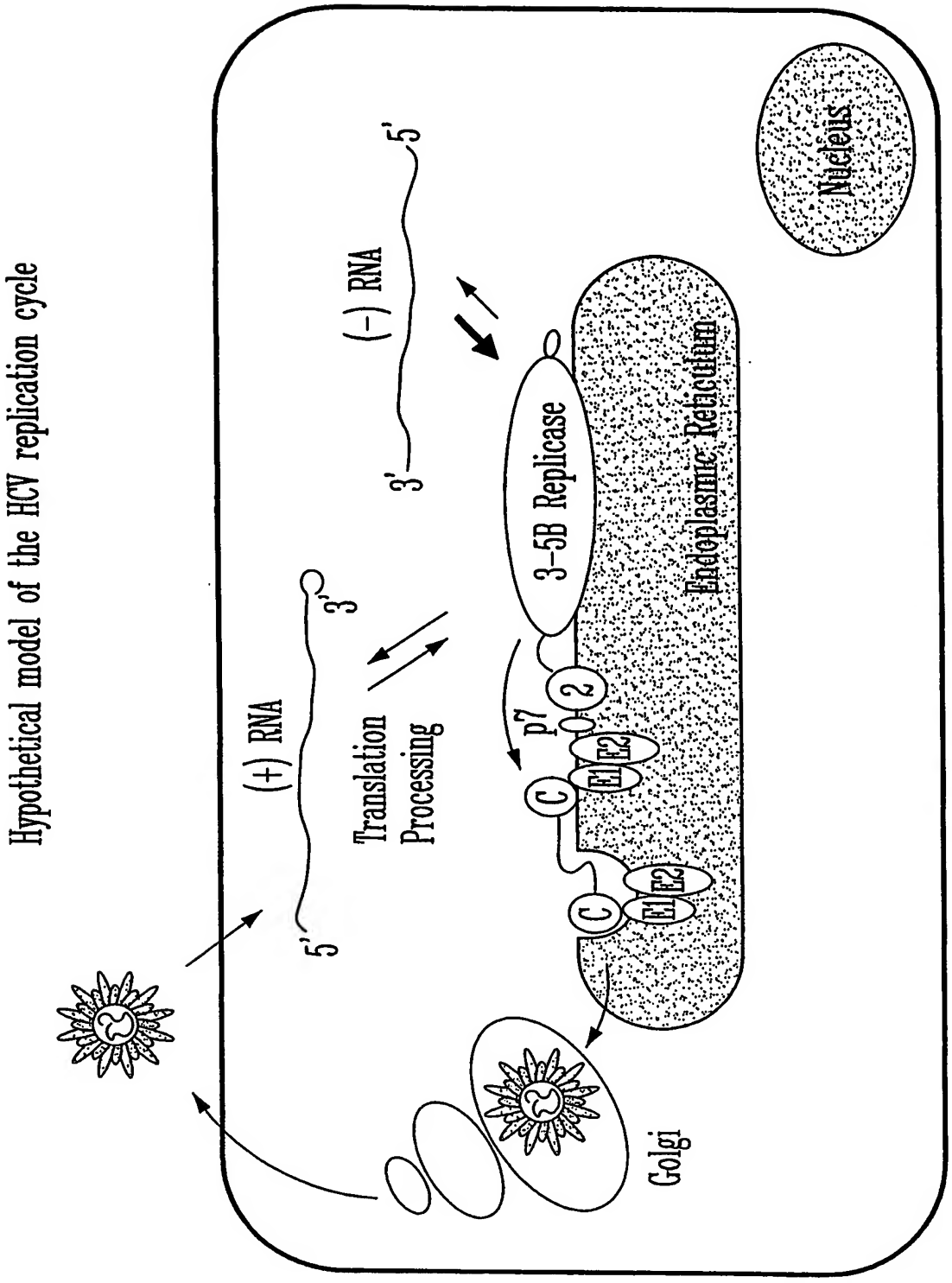

Hypothetical model of the HCV replication cycle
(+) RNA
5'
3'
Translation
Processing
(-) RNA
3'
5'
3-5B Replicase
p7
C
E1
E2
Endoplasmic Reticulum
Nucleus
Golgi

FIG. 2

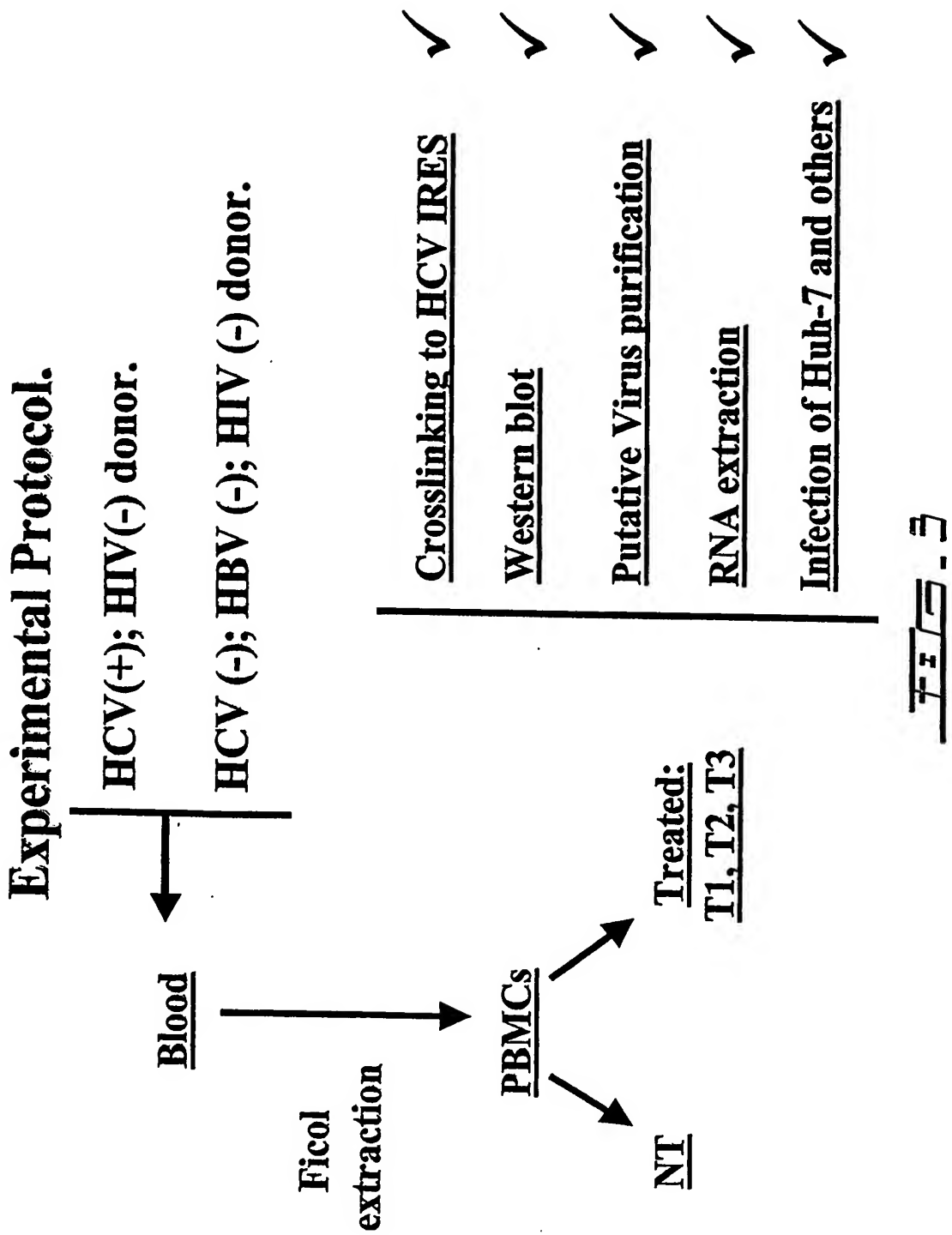

Experimental Protocol.
HCV(+); HIV(-) donor.
HCV (-); HBV (-); HIV (-) donor.
Blood
Ficol extraction
PBMCs
NT
Treated: T1, T2, T3
Crosslinking to HCV IRES
Western blot
Putative Virus purification
RNA extraction
Infection of Huh-7 and others

Fig. 3

PBMC and PBLC purification from blood samples.

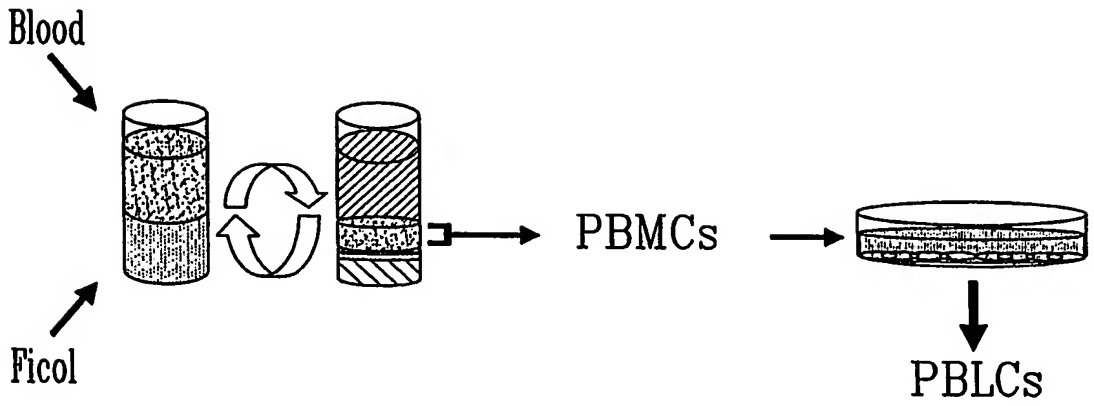


Fig. 4

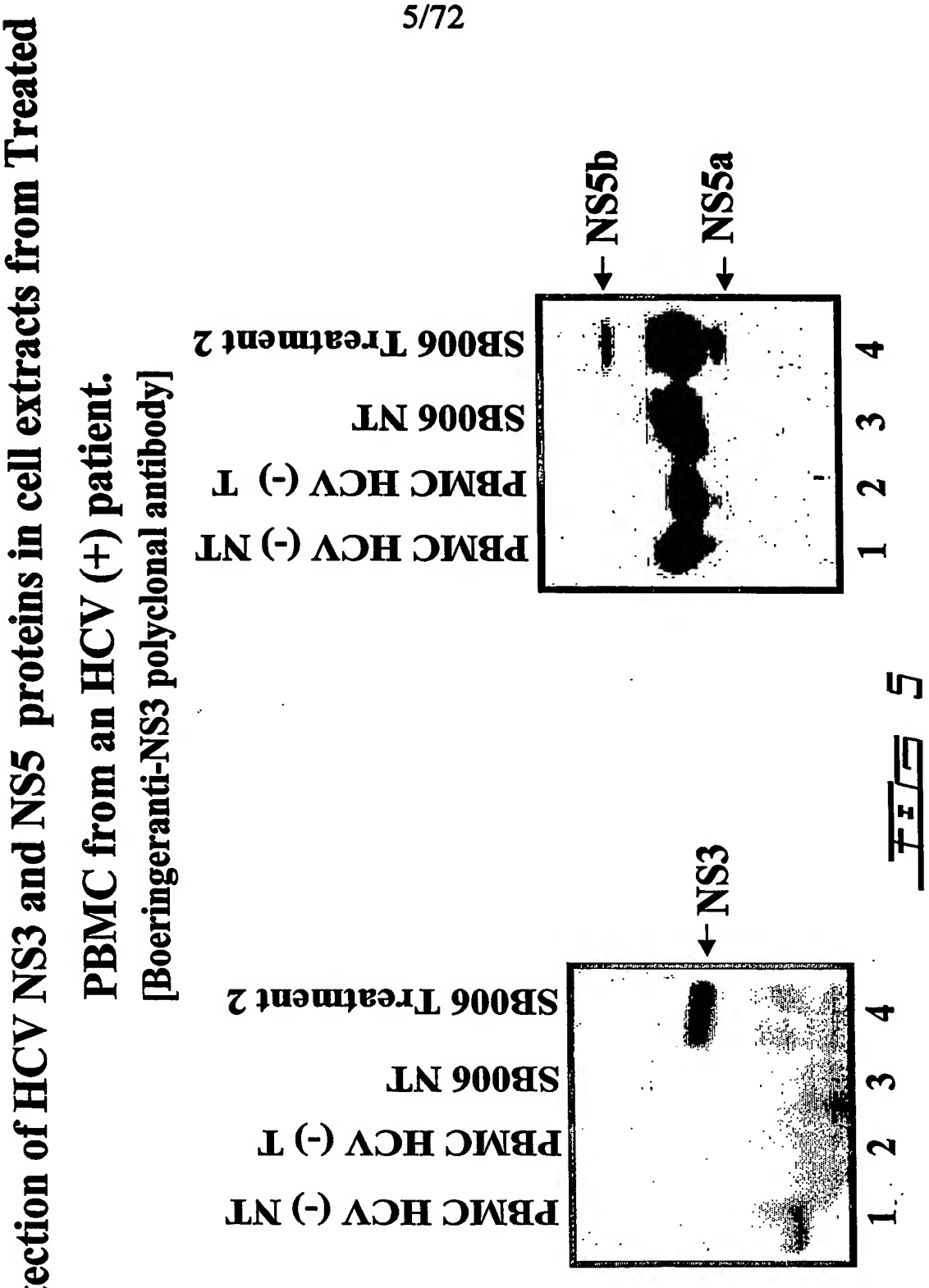

Detection of HCV NS3 and NS5 proteins in cell extracts from Treated PBMC from an HCV (+) patient.
[Boeringeranti-NS3 polyclonal antibody]
PBMC HCV (-) NT
PBMC HCV (-) T
SB006 NT
SB006 Treatment 2
1 2 3 4
NS3
NS5a
NS5b

Fig 5

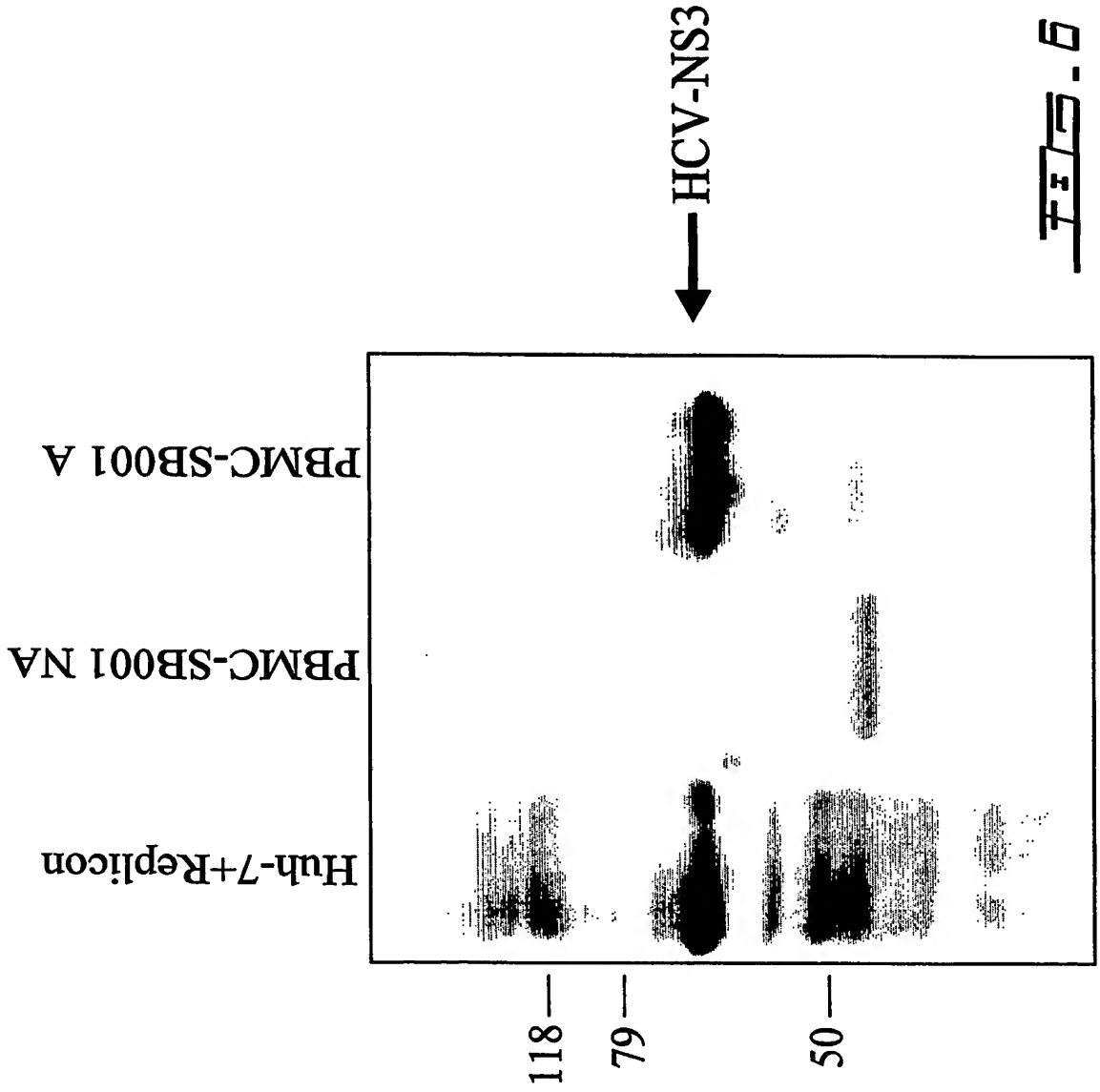


FIG. 6

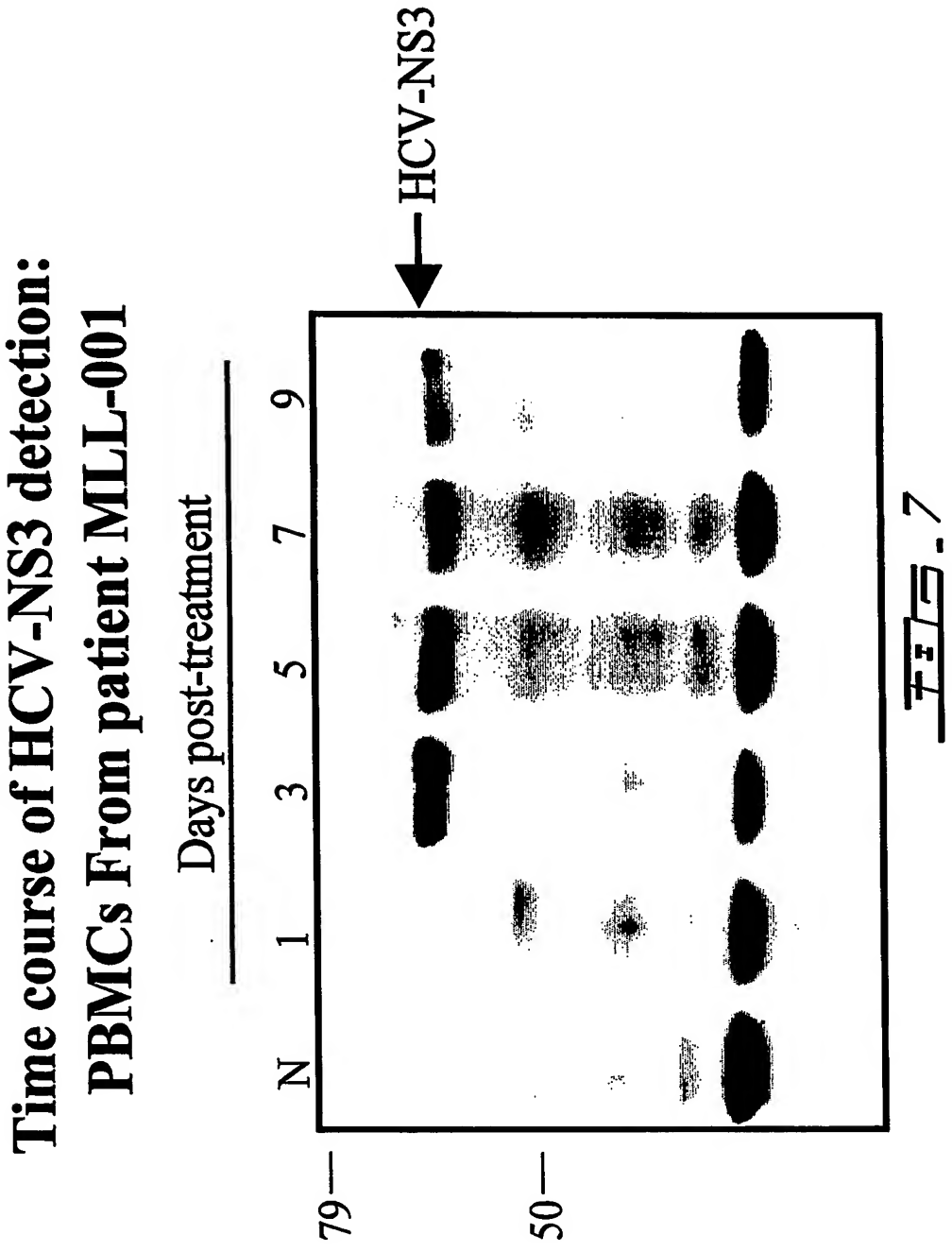

Time course of HCV-NS3 detection:
PBMCs From patient MLL-001
Days post-treatment
N
1
3
5
7
9
HCV-NS3
79
50

Fig. 7

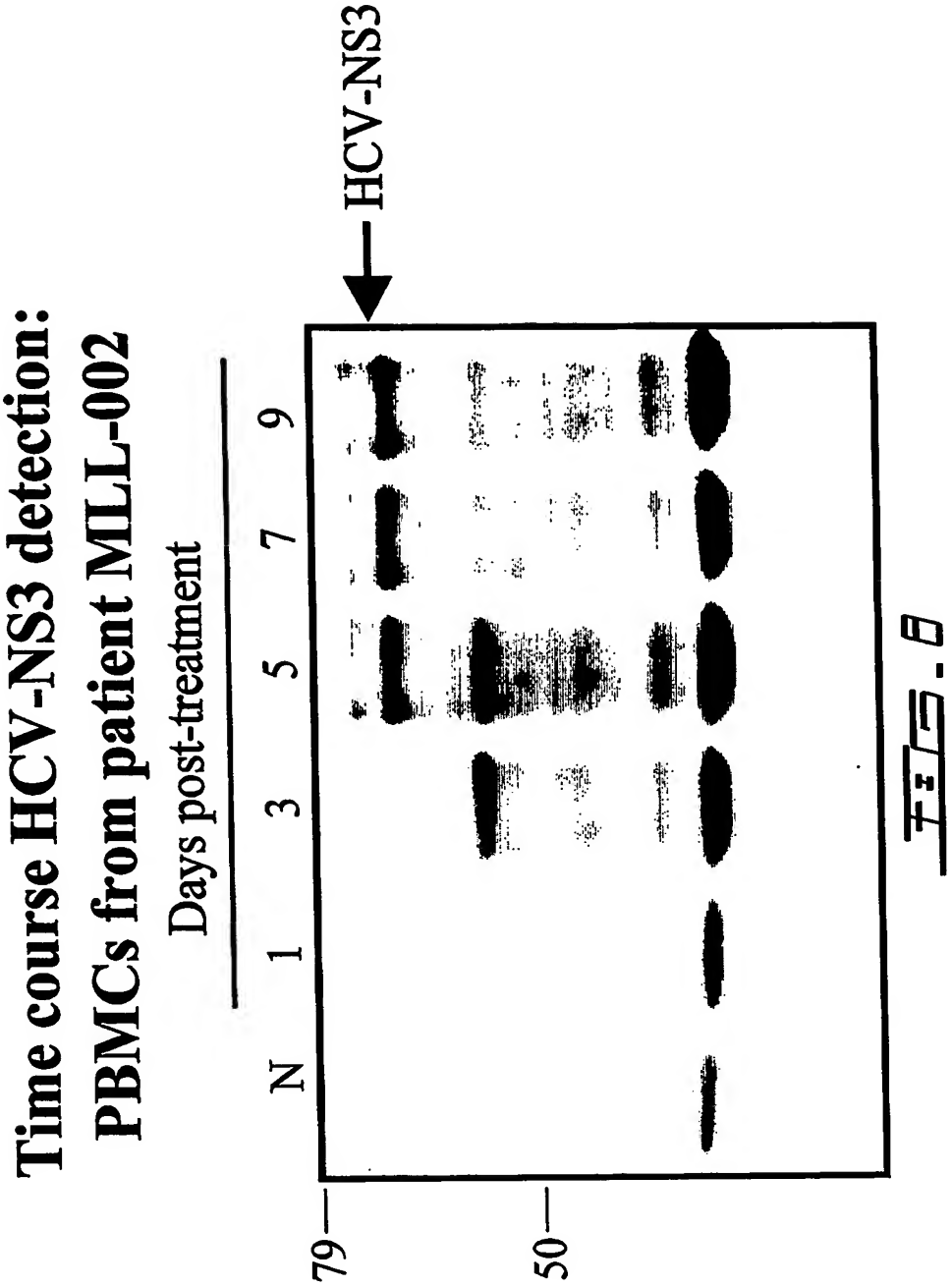

Time course HCV-NS3 detection:
PBMCs from patient MLL-002
Days post-treatment
N
1
3
5
7
9
HCV-NS3
79
50

Fig. 8

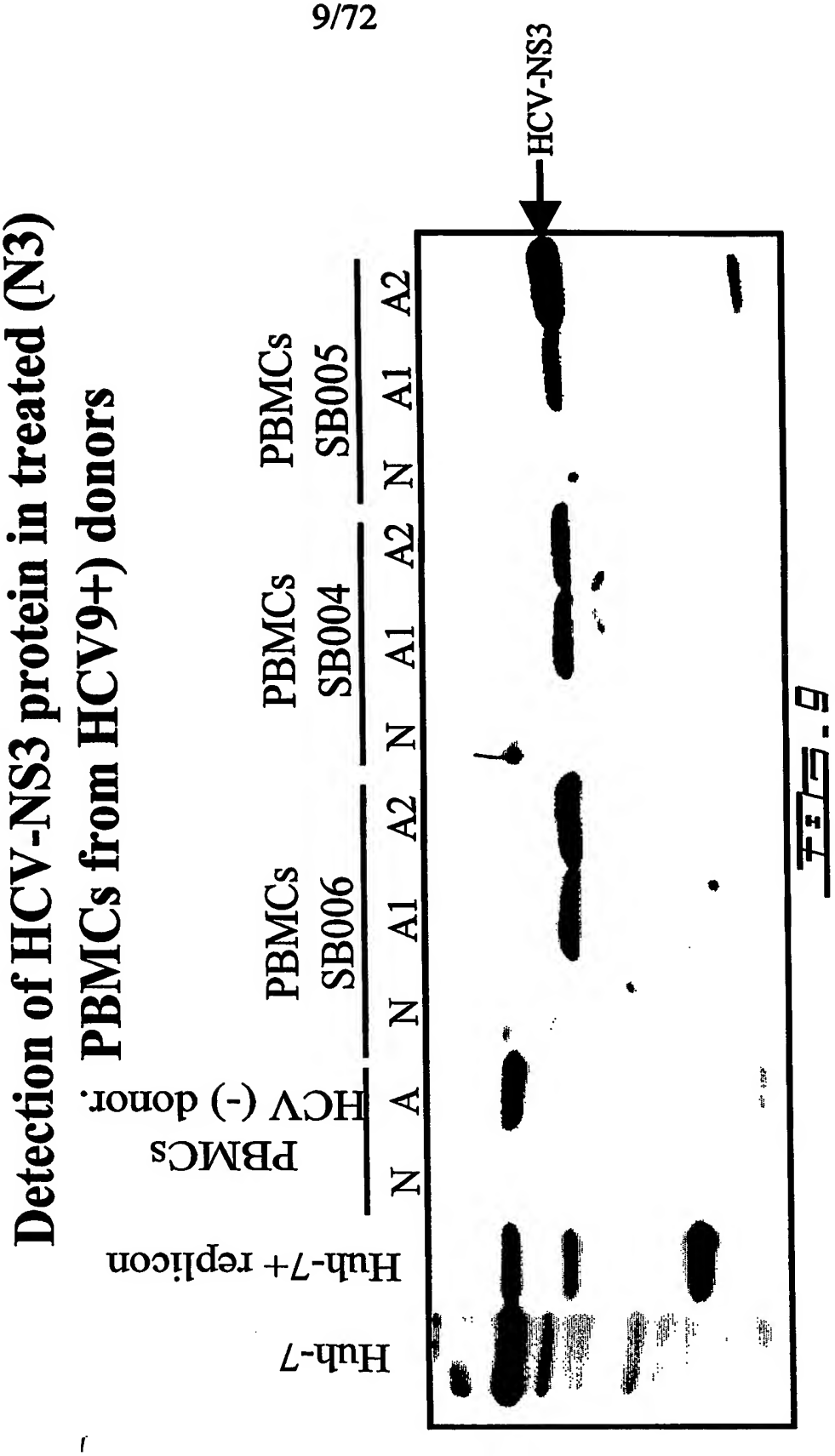
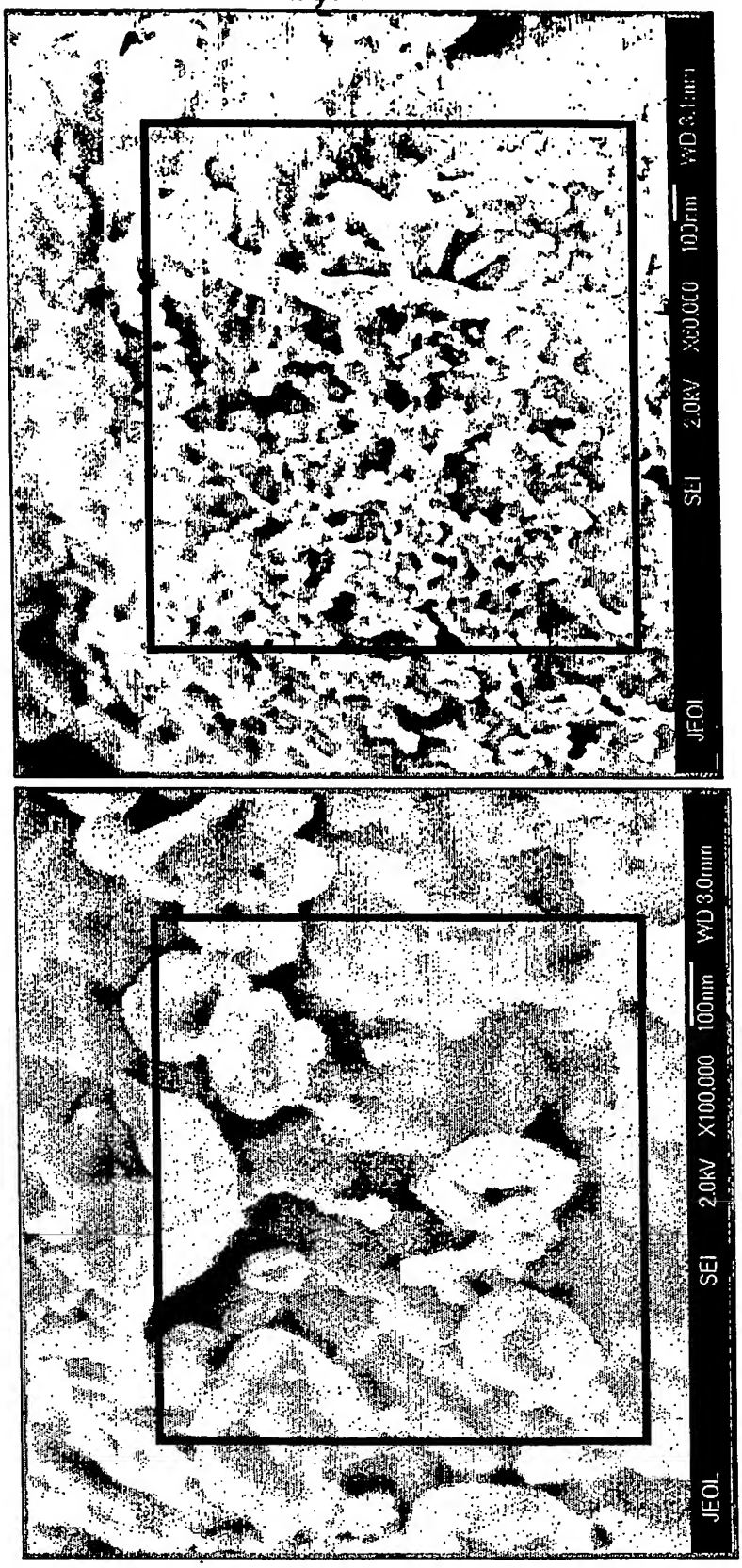

Detection of HCV-NS3 protein in treated (N3) PBMCs from HCV9+) donors
HCV-NS3
Huh-7
Huh-7+ replicon
PBMCs HCV (-) donor.
N A N A1 A2
PBMCs SB006
N A1 A2
PBMCs SB004
N A1 A2
PBMCs SB005
N A1 A2

Fig. 9

Detection of virus like particles by scanning electron microscopy



(-) Control

FIG. 10

Electron microscopy of Activated PBLCs;
Detection of virus like particles

Fig. 11

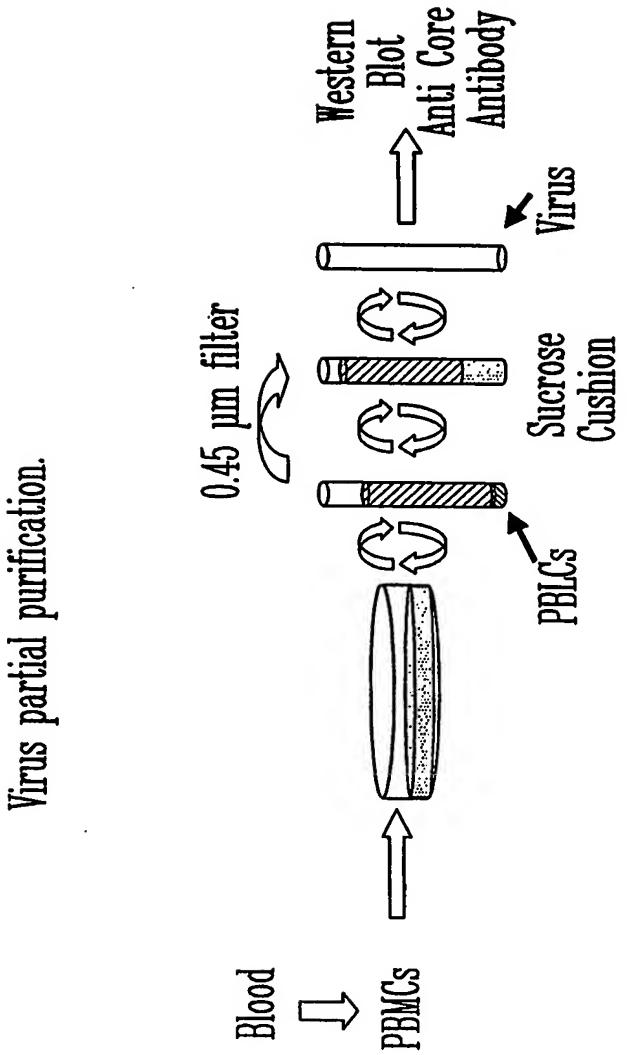

Virus partial purification.
Blood
PBMCs
PBLCs
0.45 µm filter
Sucrose Cushion
Virus
Western Blot Anti Core Antibody

FIG. 12

Detection of HCV Core protein in supernatant of treated PBMC from an HCV (+) patient.
[Maine biotechnology anti-Core monoclonal antibody]

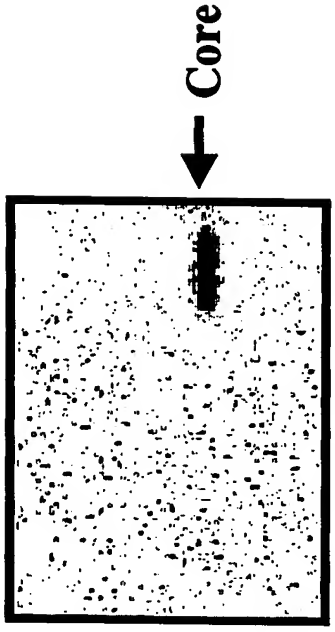


Fig. 13

SUBSTITUTE SHEET (RULE 26)

RNA Quantification I (virus copies/ng total RNA)

Patient	HCV RNA In PBMC	Detection of Core (wb) in supernatant
After 4 days		
SB004 NT	$2x10^3$	No
SB004 T	$2x10^3$	Yes
SB006 NT	$1.8 x10^3$	No
SB006 T	$2x10^2$	Yes
After 20 days		
SB004	0.00	
SB006	0.00	

Fig. 14

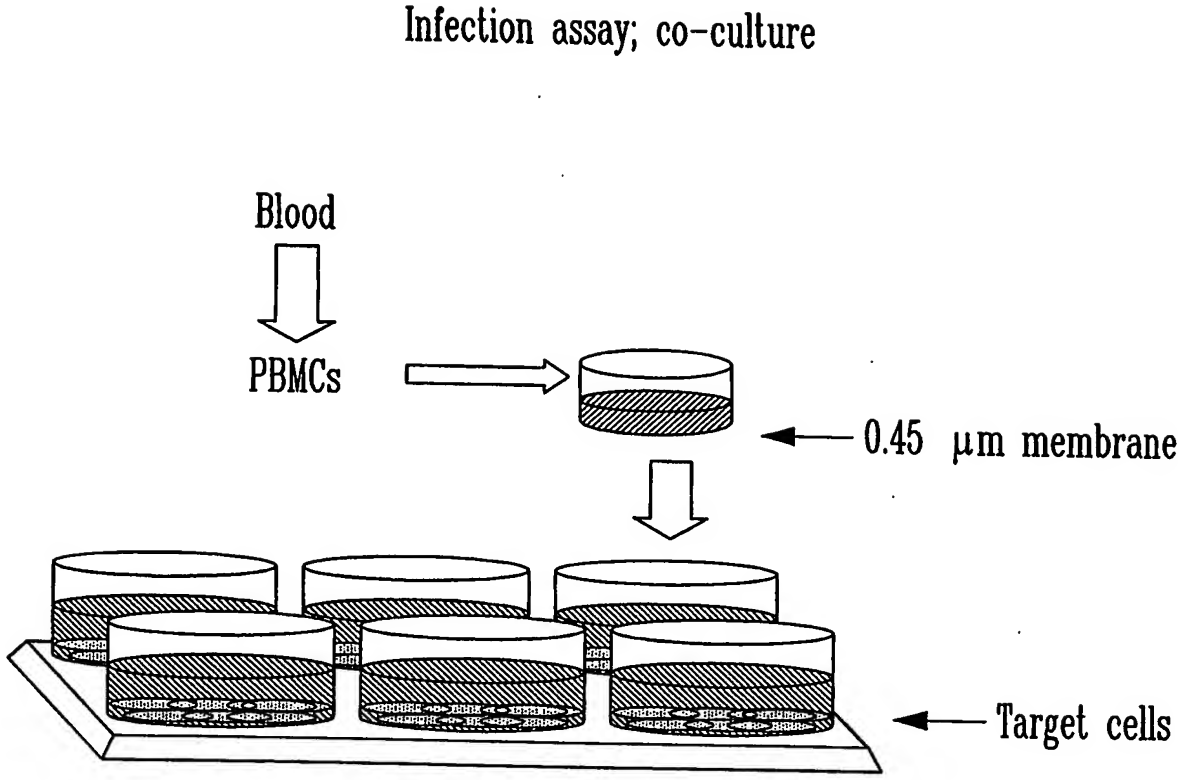

Infection assay; co-culture
Blood
PBMCs
0.45 µm membrane
Target cells

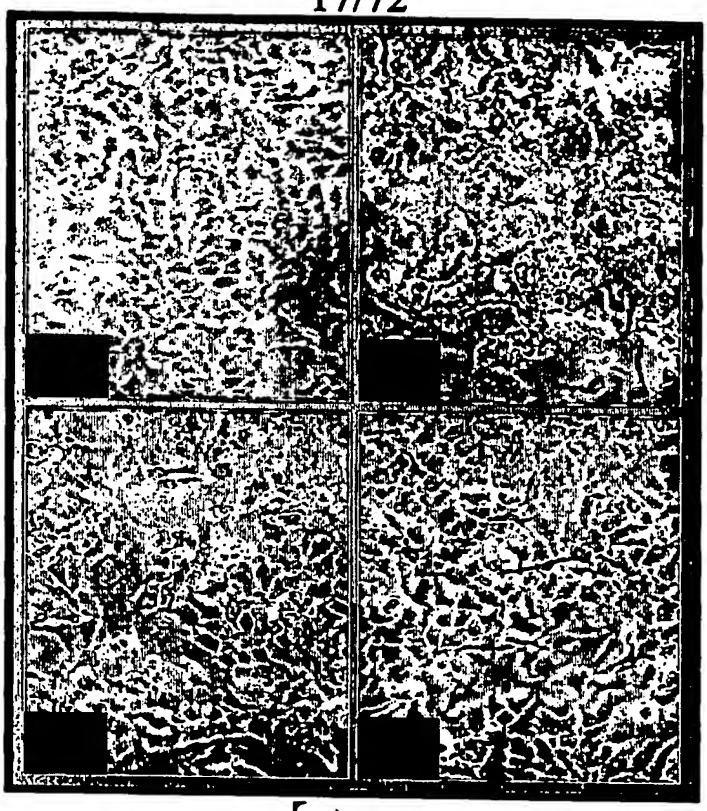
FIG_15

SUBSTITUTE SHEET (RULE 26)

Infection of MT-4 cells
RNA Quantification II (virus copies/ng total RNA)

Patient	HCV RNA In PBMC	Detection of Core (wb) in supernatant	HCV RNA In MT-4
After 10 days			
SB001 NT	13	No	0.00
SB001 T	12	Yes	1600
After 20 days			
SB001	0.00		0.00
SB001	0.00		0.00

Fig. 16

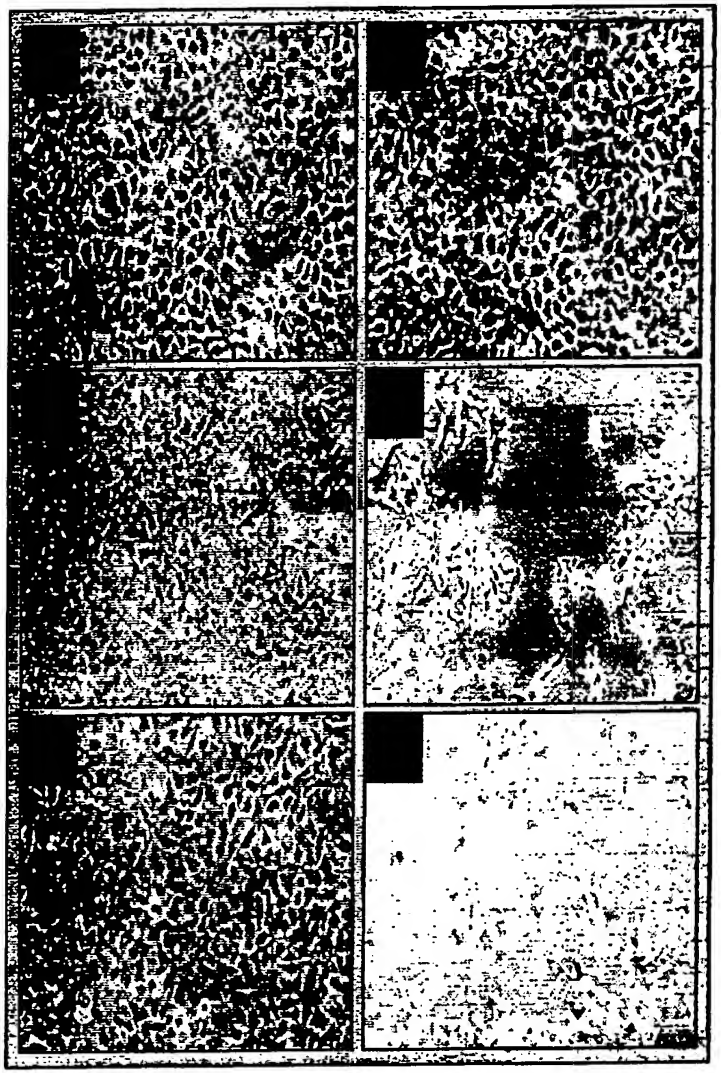


Co-culture of Huh-7 and HCV (-) PBMCs.

1- Huh-7
2- Huh-7 + PBMCs HCV (-) NT
3- Huh-7 + Treatment
4- Huh-7 + PBMCs HCV (-) T

Fig. 17

Co-culture of Huh-7 and HCV (+) PBMS° Cs (SB006).



1. Huh-7
2-3. Huh-7 + PBMCs HCV (+) NT
4. Huh-7 + Treatment
5-6. Huh-7 + PBMCs HCV (+) T

Fig. 18

SUBSTITUTE SHEET (RULE 26)

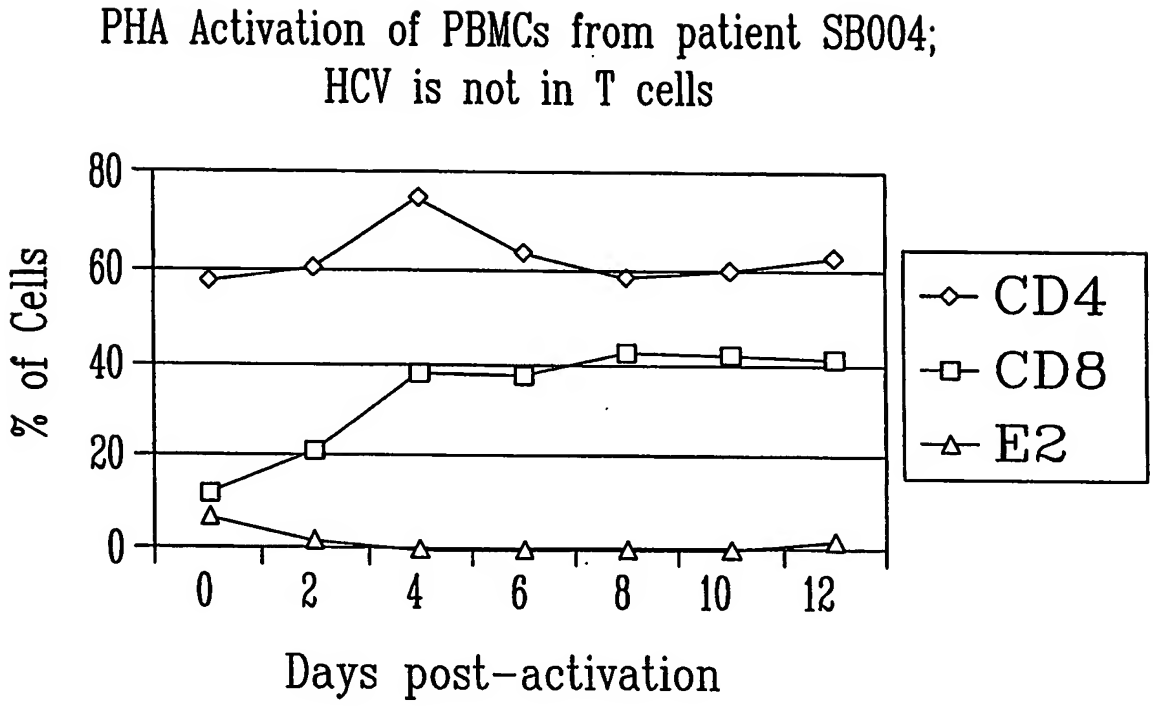

PHA Activation of PBMCs from patient SB004;
HCV is not in T cells
% of Cells
80
60
40
20
0
0
2
4
6
8
10
12
Days post-activation
CD4
CD8
E2

FIG. 19

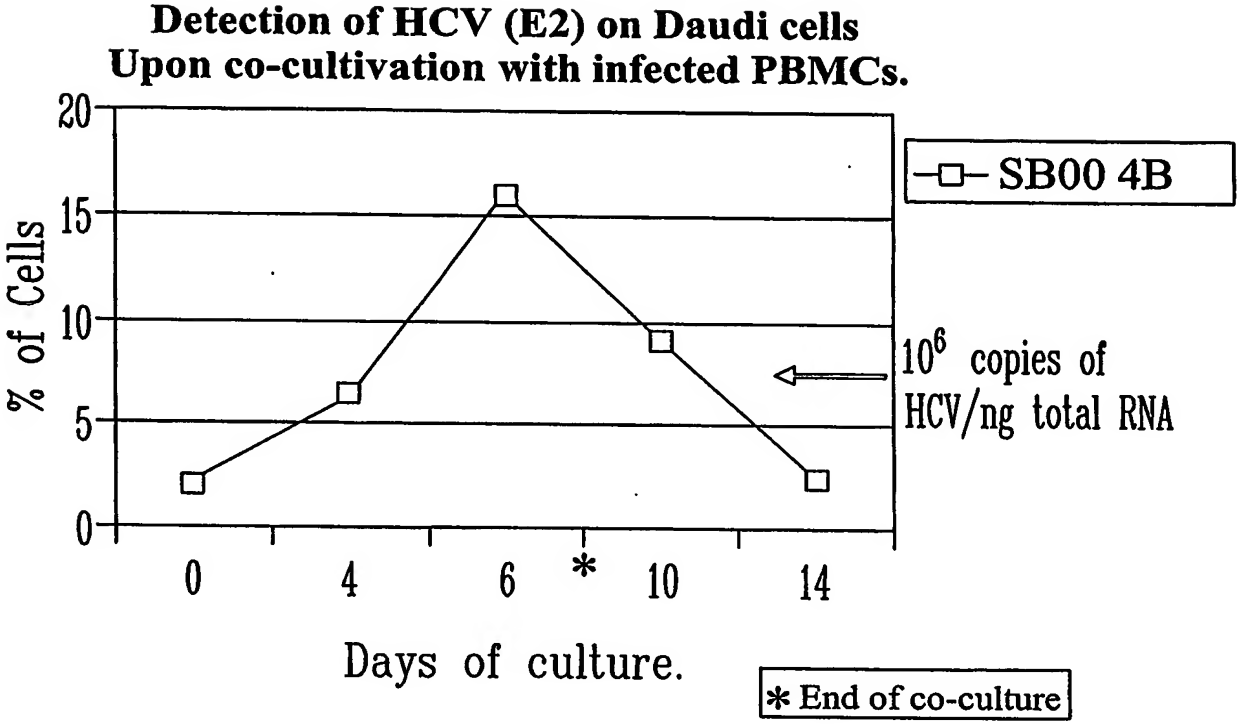

Detection of HCV (E2) on Daudi cells
Upon co-cultivation with infected PBMCs.
20
15
10
5
0
% of Cells
SB00 4B
10^6 copies of
HCV/ng total RNA
0
4
6
*
10
14
Days of culture.
* End of co-culture

FIG. 20

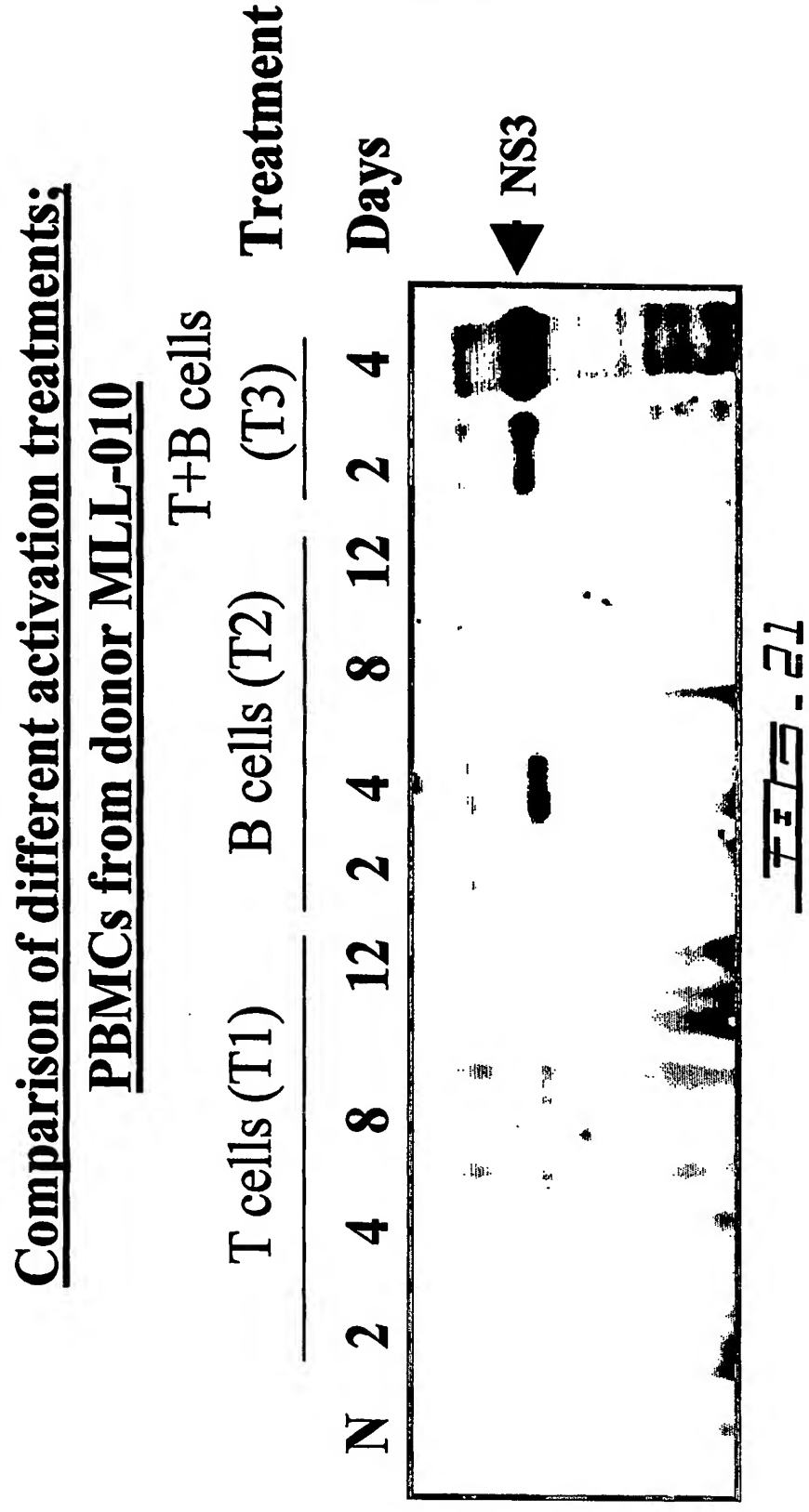

Comparison of different activation treatments;
PBMCs from donor MLL-010
T cells (T1)
B cells (T2)
T+B cells (T3)
Treatment
N 2 4 8 12 2 4 8 12 2 4
Days
NS3

Fig. 21

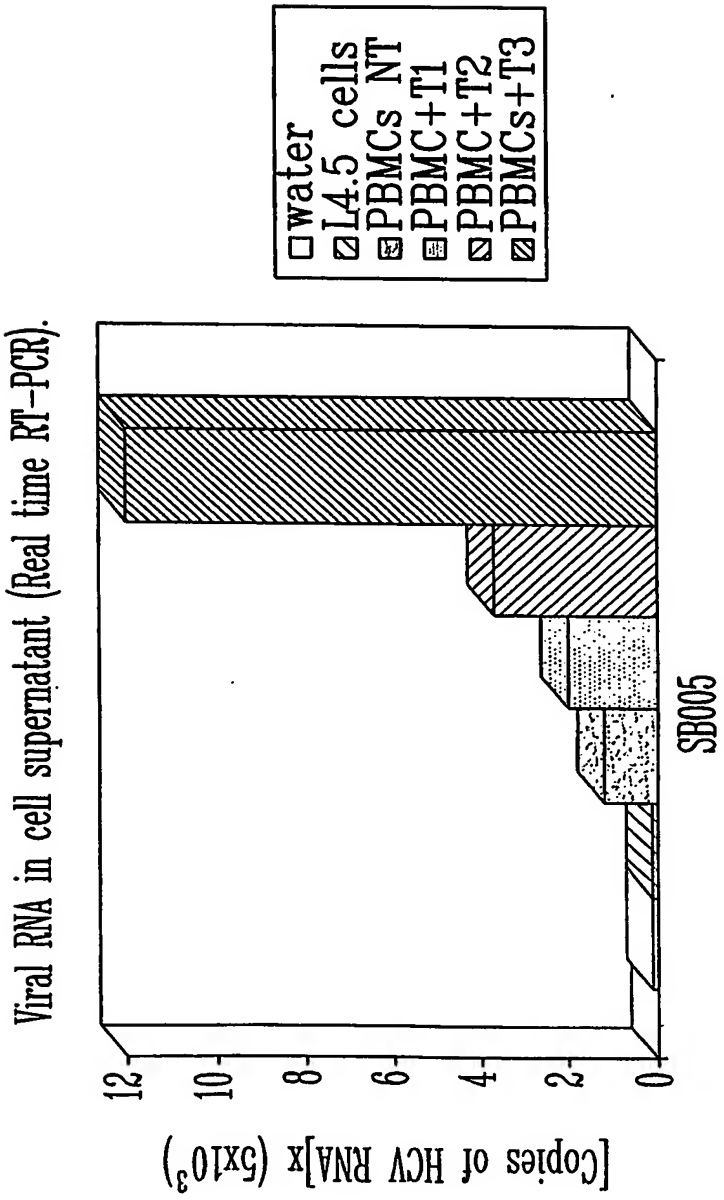

Viral RNA in cell supernatant (Real time RT-PCR).
[Copies of HCV RNA]x (5x10³)
0
2
4
6
8
10
12
SB005
water
L4.5 cells
PBMCs NT
PBMC+T1
PBMC+T2
PBMCs+T3

Fig. 22

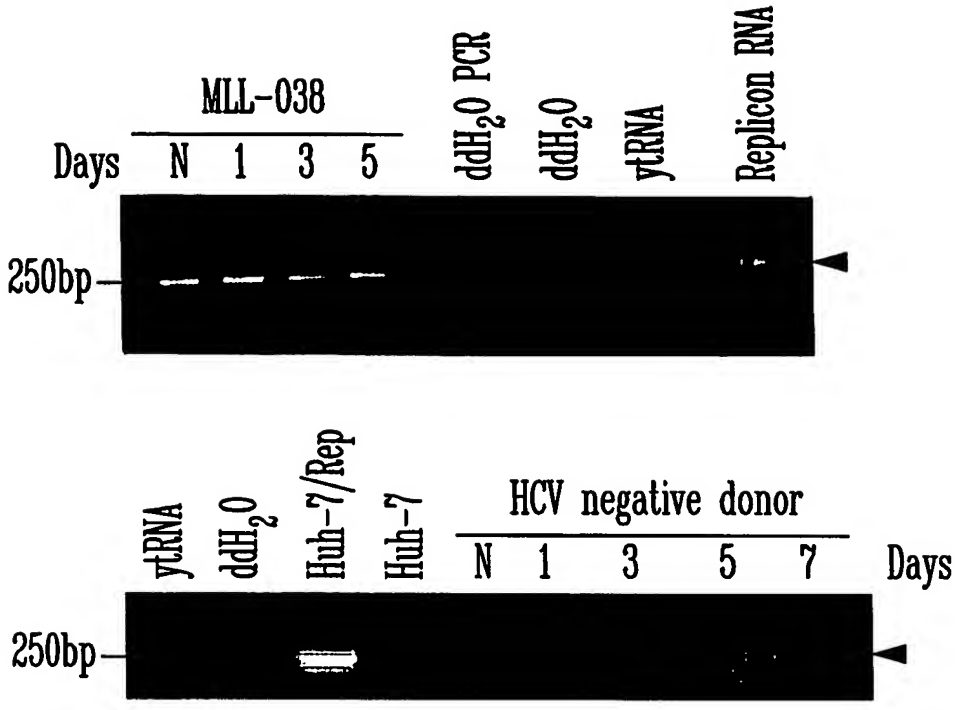


Fig_23A

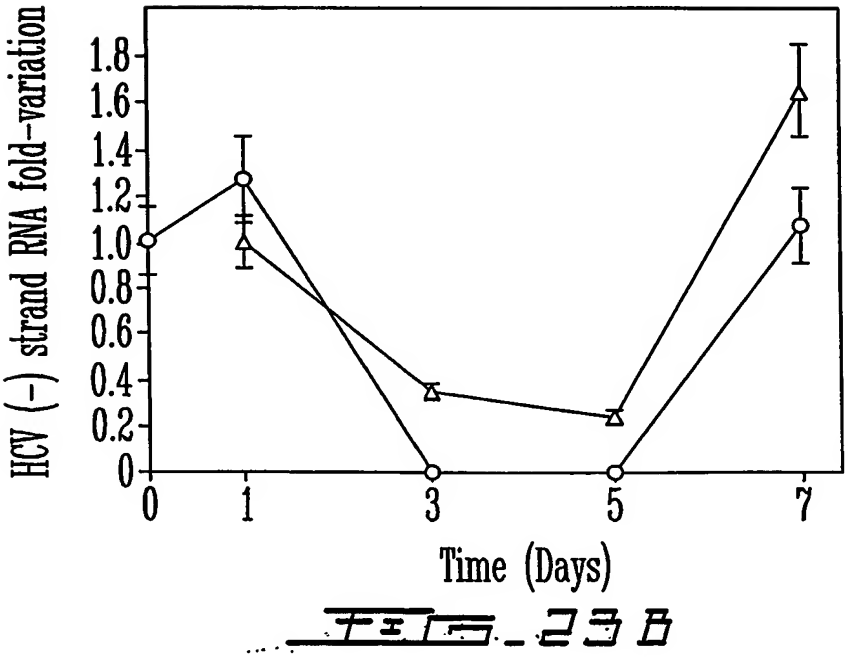


Fig_23B

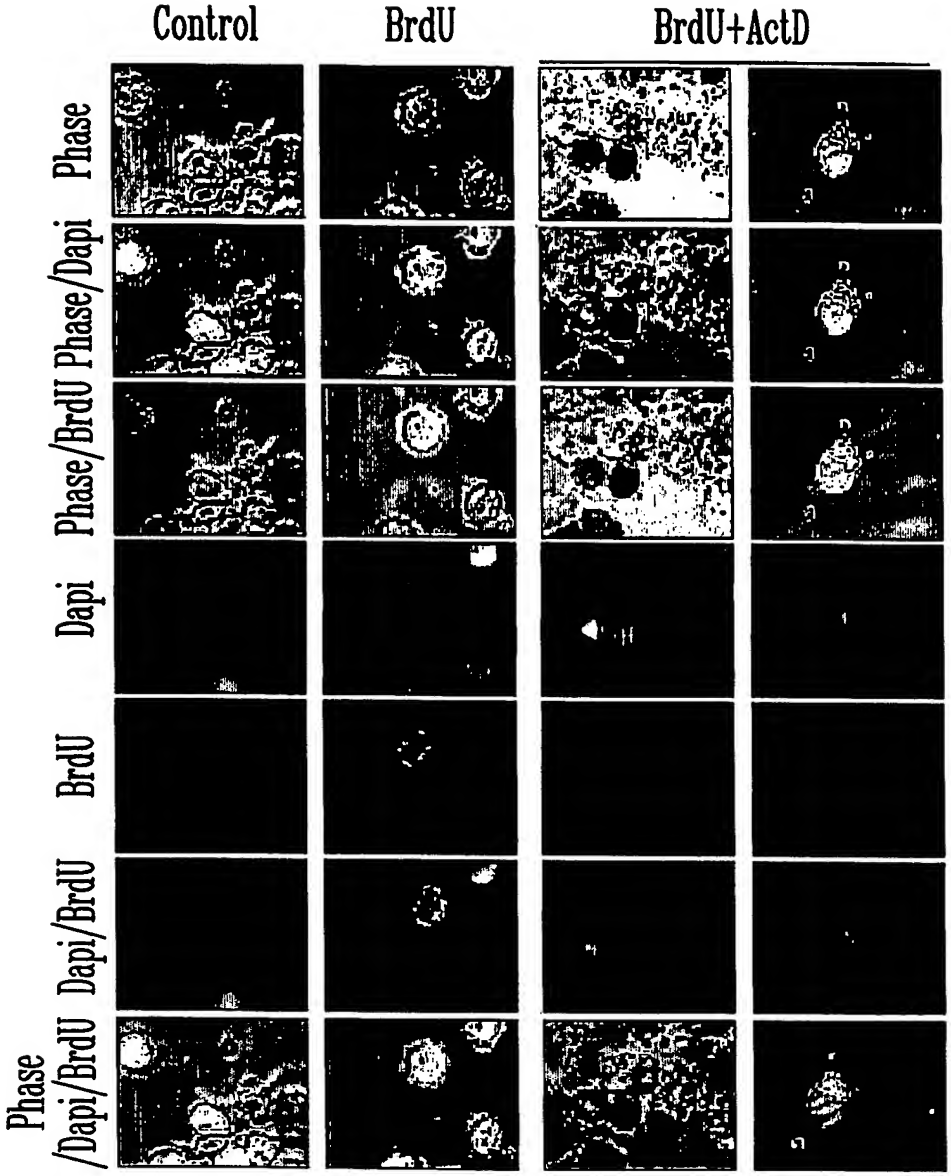

Control
BrdU
BrdU+ActD
Phase
Phase/Dapi
Phase/BrdU
Dapi
BrdU
Dapi/BrdU
Phase /Dapi/BrdU

FIG-23C

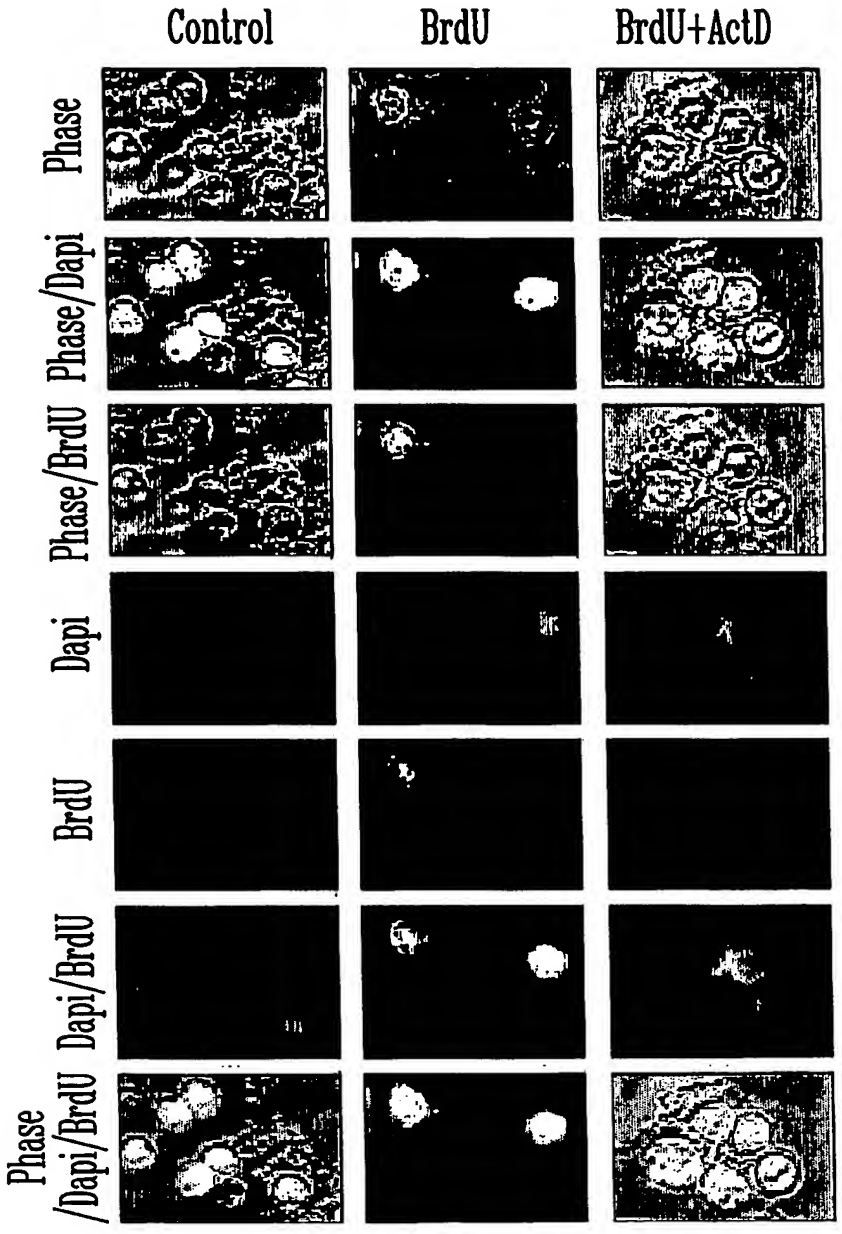

Control
BrdU
BrdU+ActD
Phase
Phase/Dapi
Phase/BrdU
Dapi
BrdU
Dapi/BrdU
Phase /Dapi/BrdU

FIG-2C

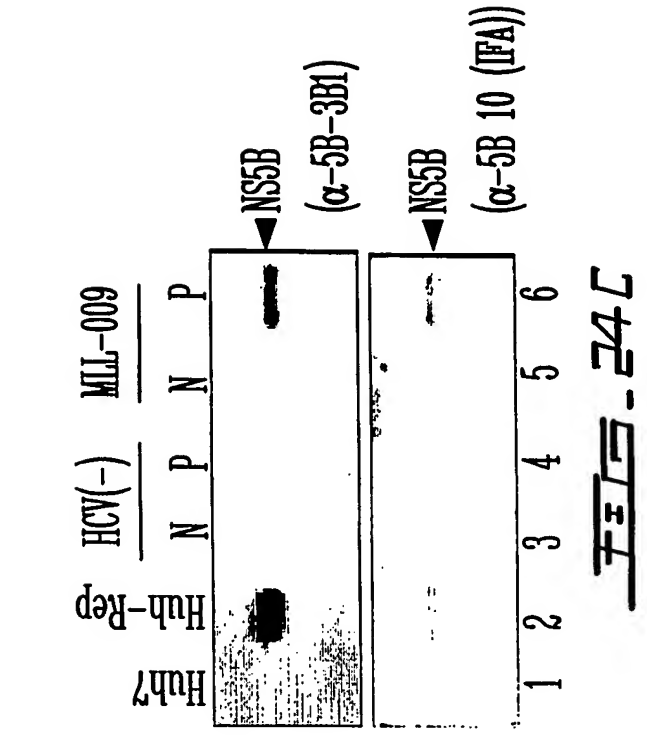


Fig. 24C

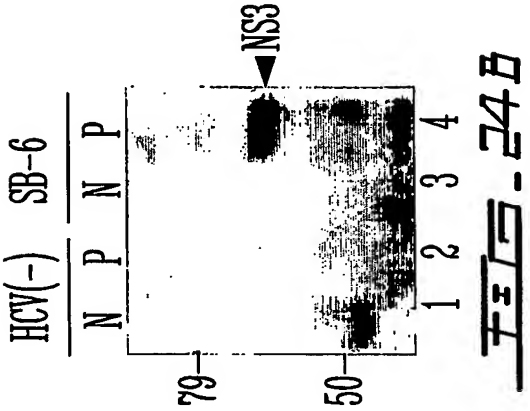


Fig. 24B

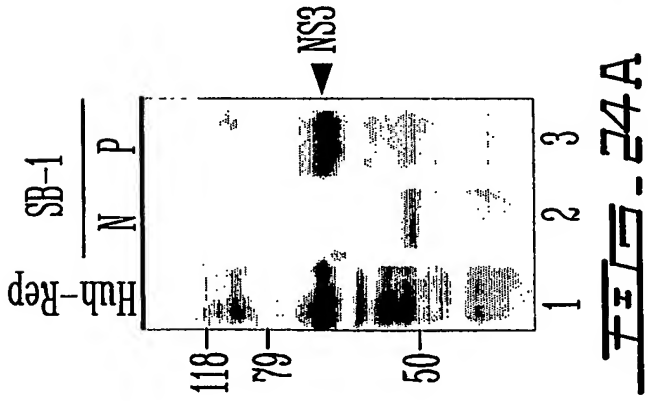


Fig. 24A

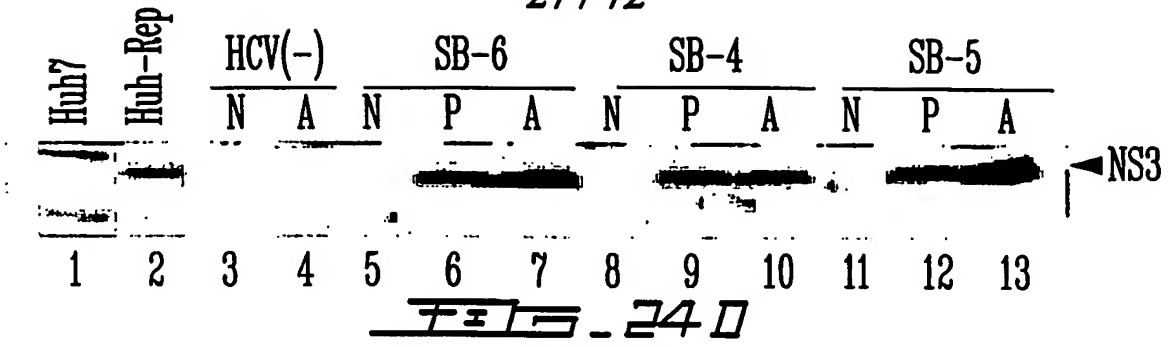


Fig_24D

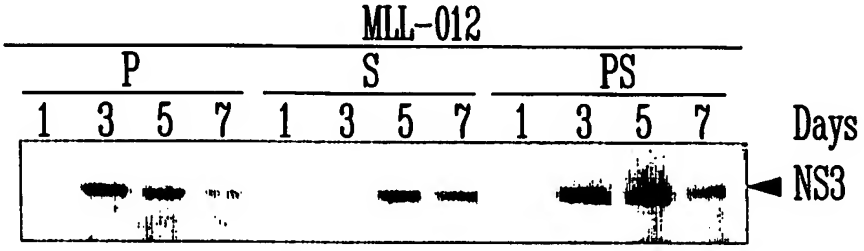


Fig_24E

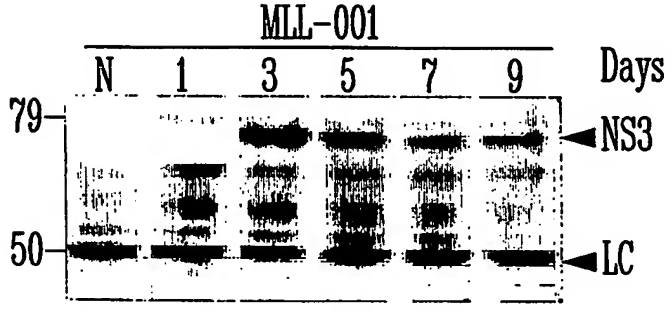


Fig_24F

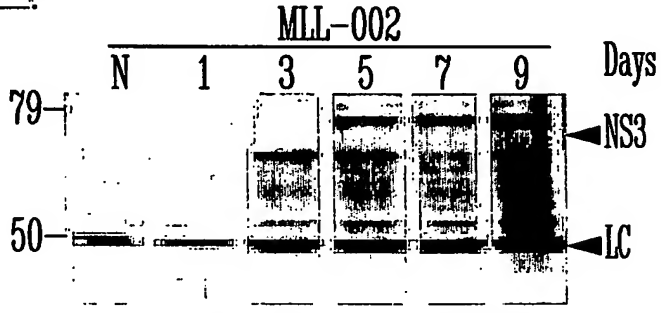


Fig_24G

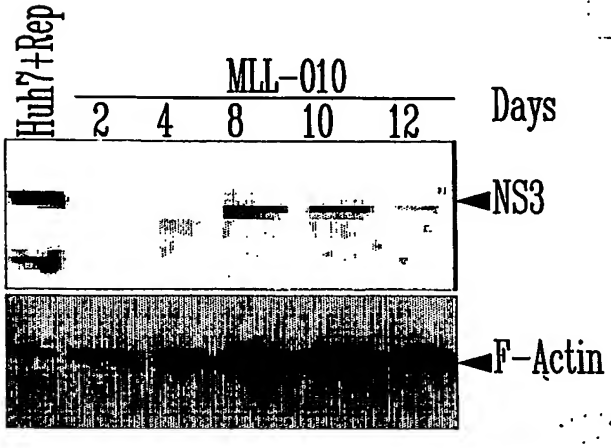


Fig_24H

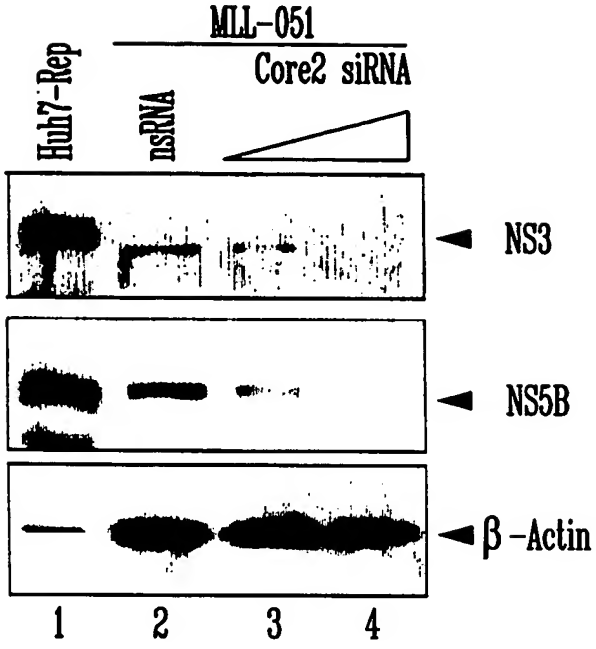


Fig_24I

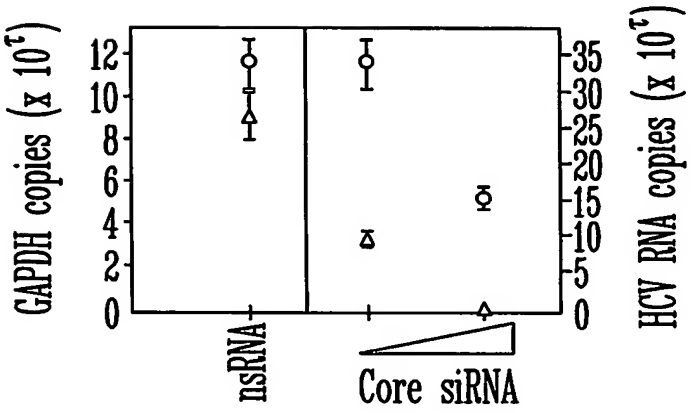


Fig_24J

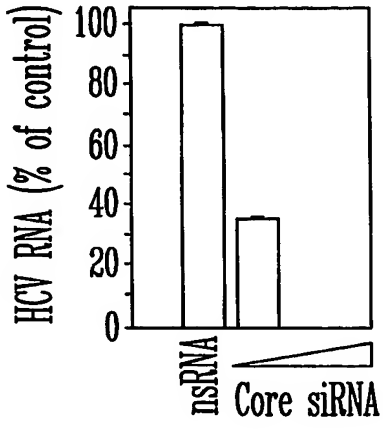


Fig_24K

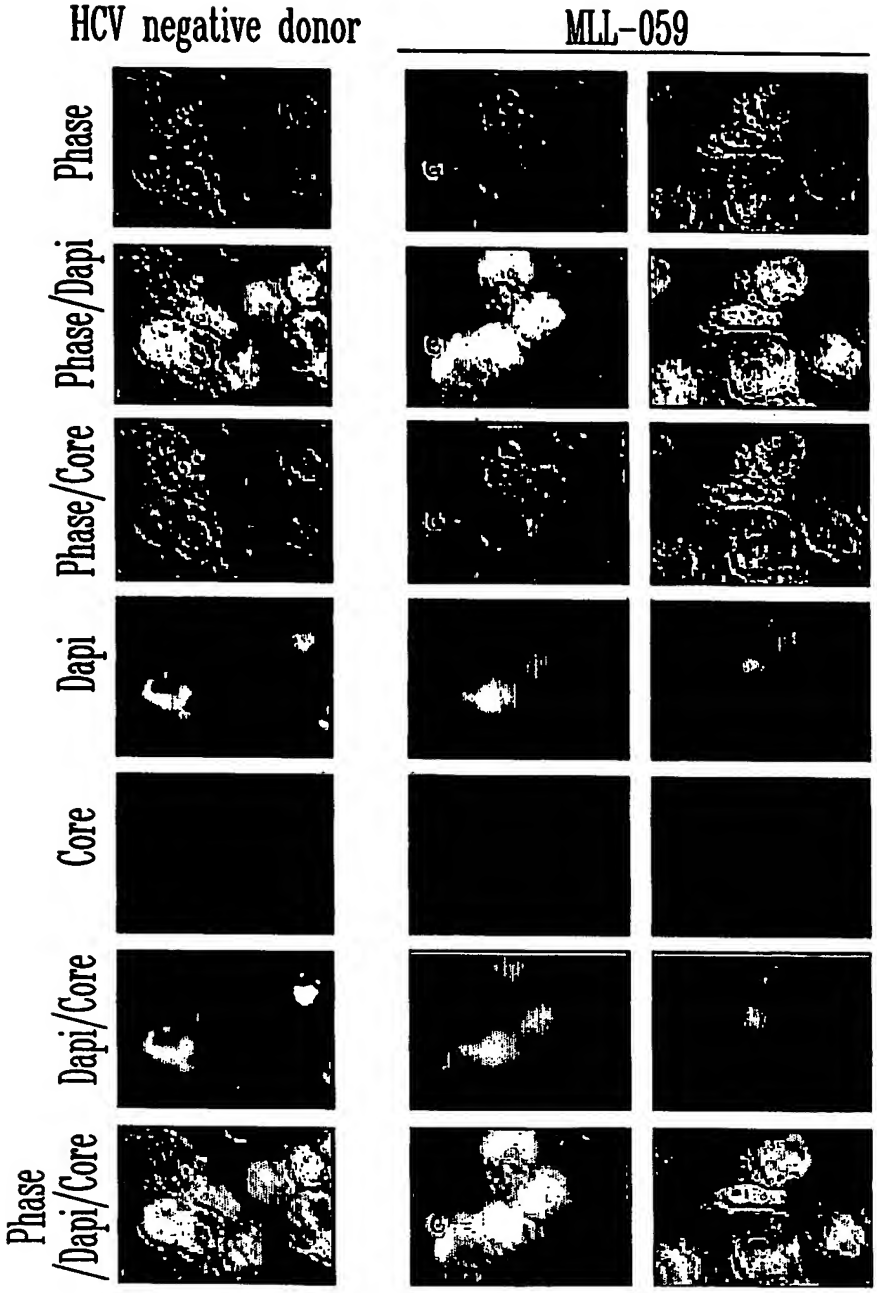

HCV negative donor
MLL-059
Phase
Phase/Dapi
Phase/Core
Dapi
Core
Dapi/Core
Phase /Dapi/Core

FIG - 25

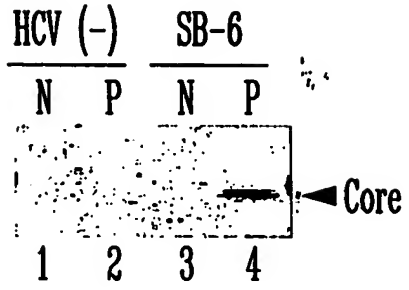


Fig. 26A

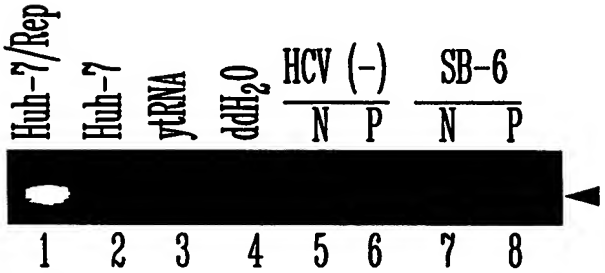


Fig. 26B

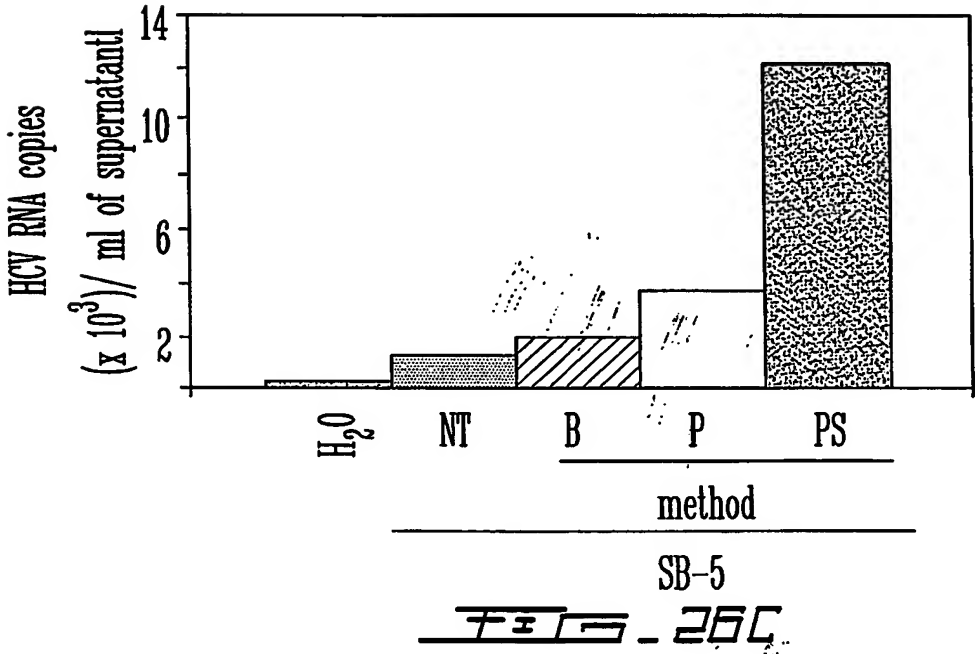


Fig. 26C

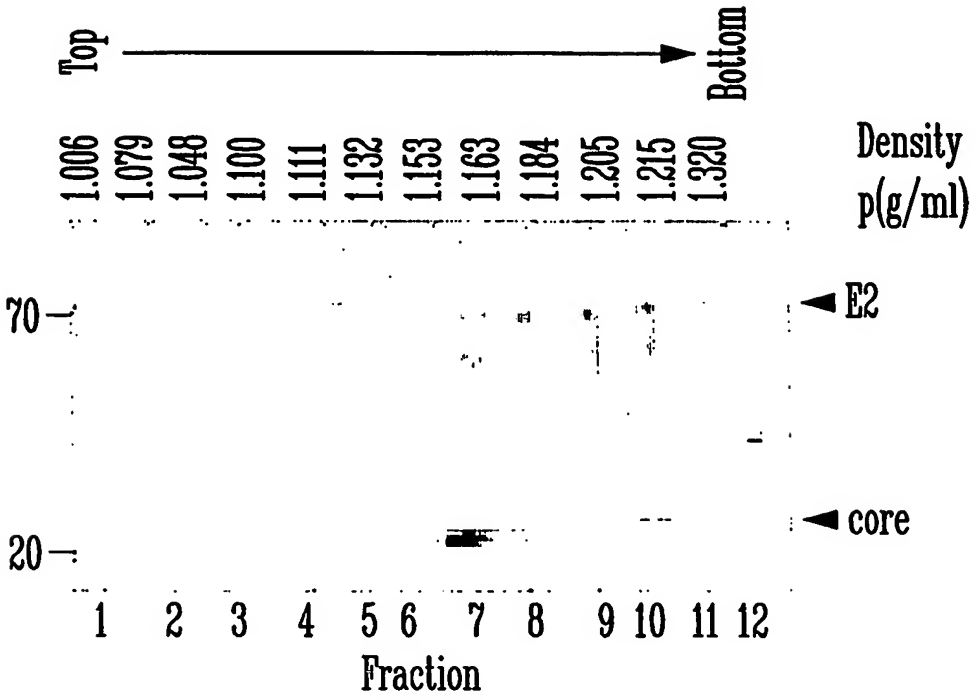


Fig. 26D

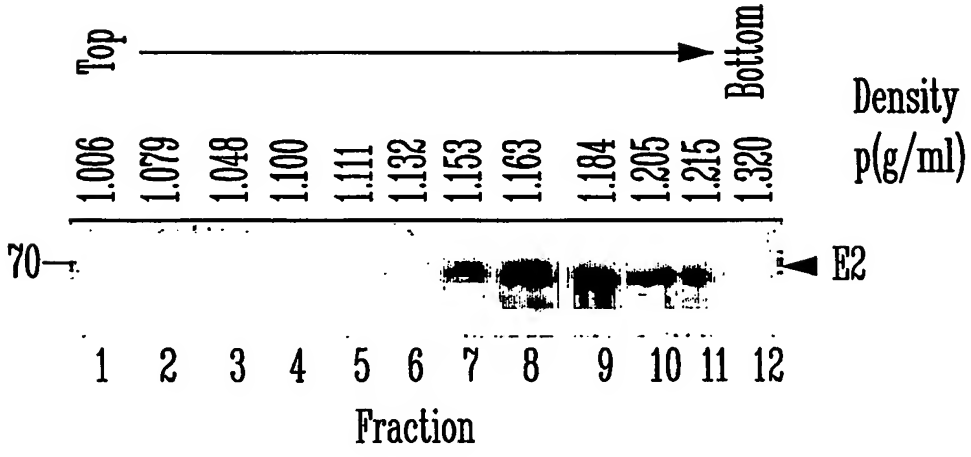


Fig. 26E

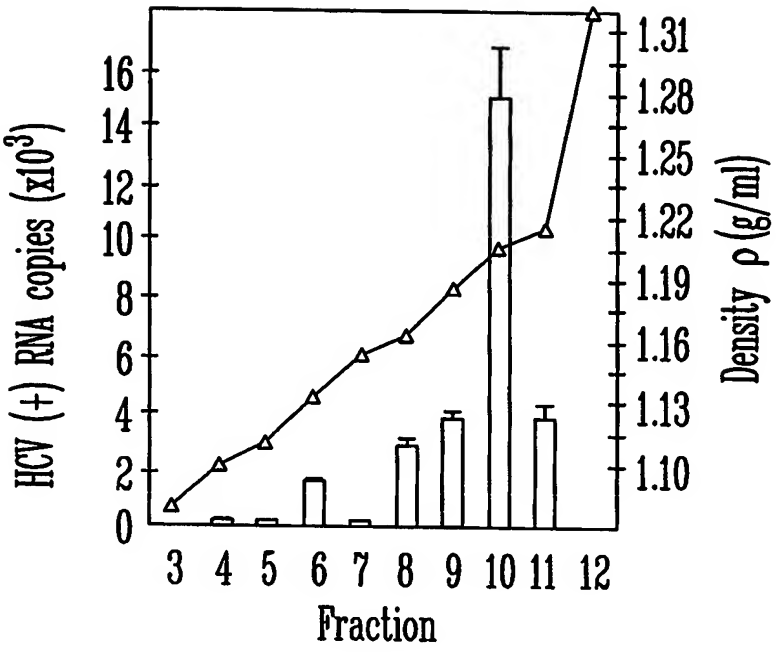


FIG. 26F

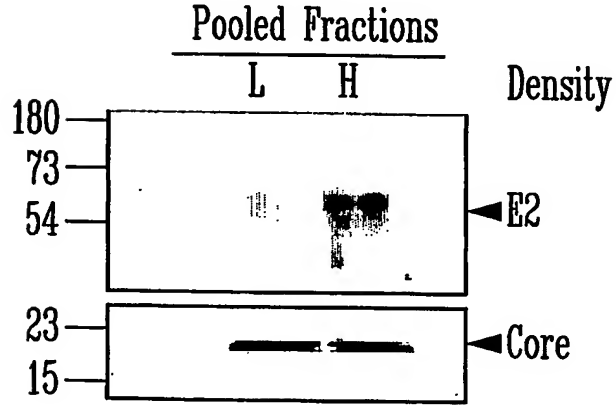


FIG. 26G

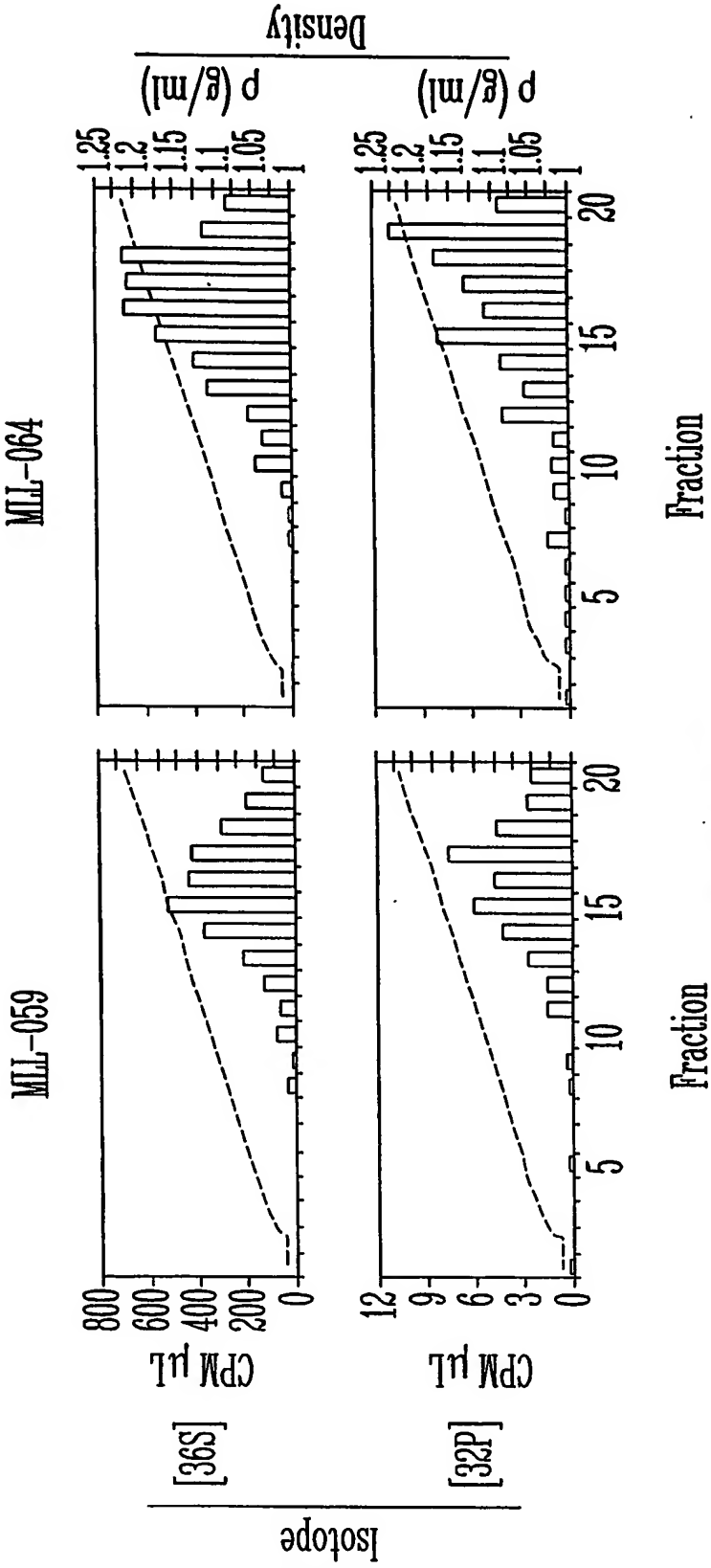

Isotope
[35S]
[32P]
CPM µL
CPM µL
0
200
400
600
800
0
3
6
9
12
MLL-059
MLL-064
Fraction
Fraction
5
10
15
20
Density
ρ (g/ml)
ρ (g/ml)
1
1.05
1.1
1.15
1.2
1.25

FIG. 2BH

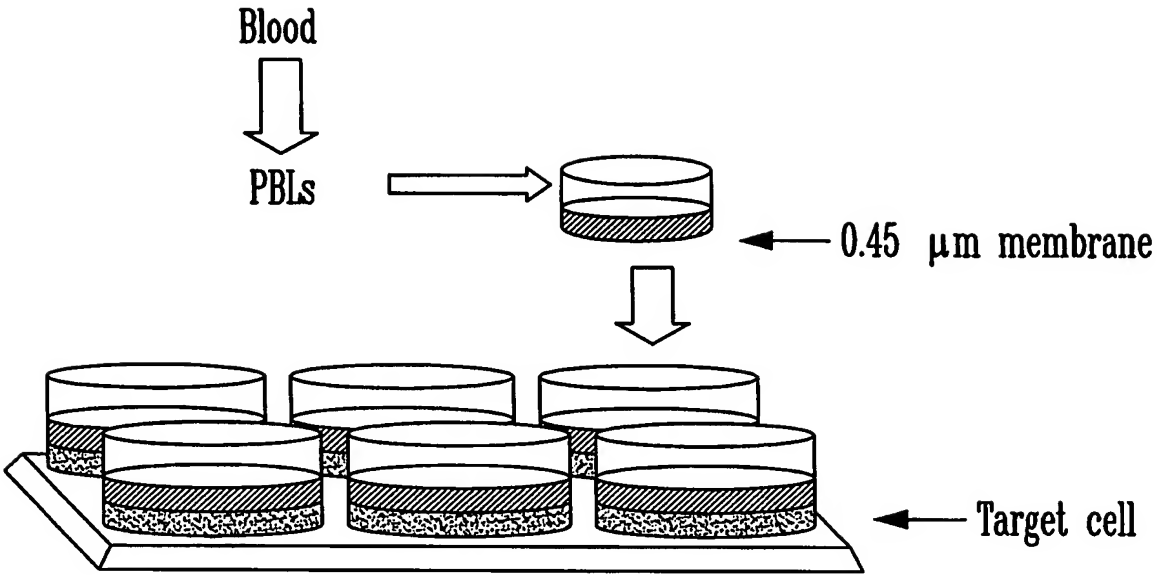


FIG_27A

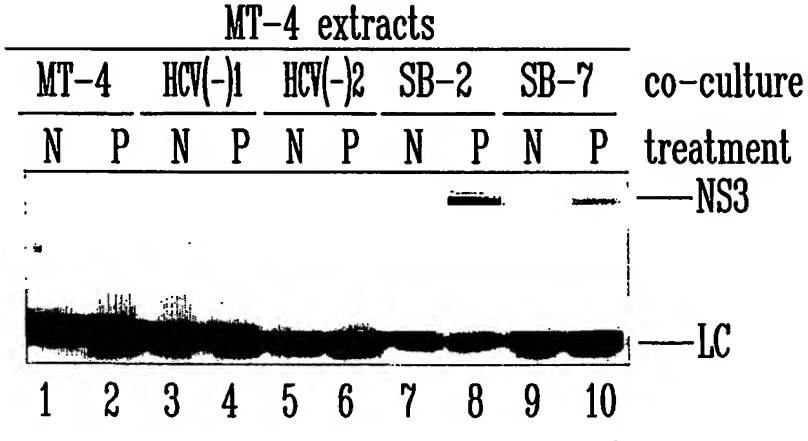


FIG_27B

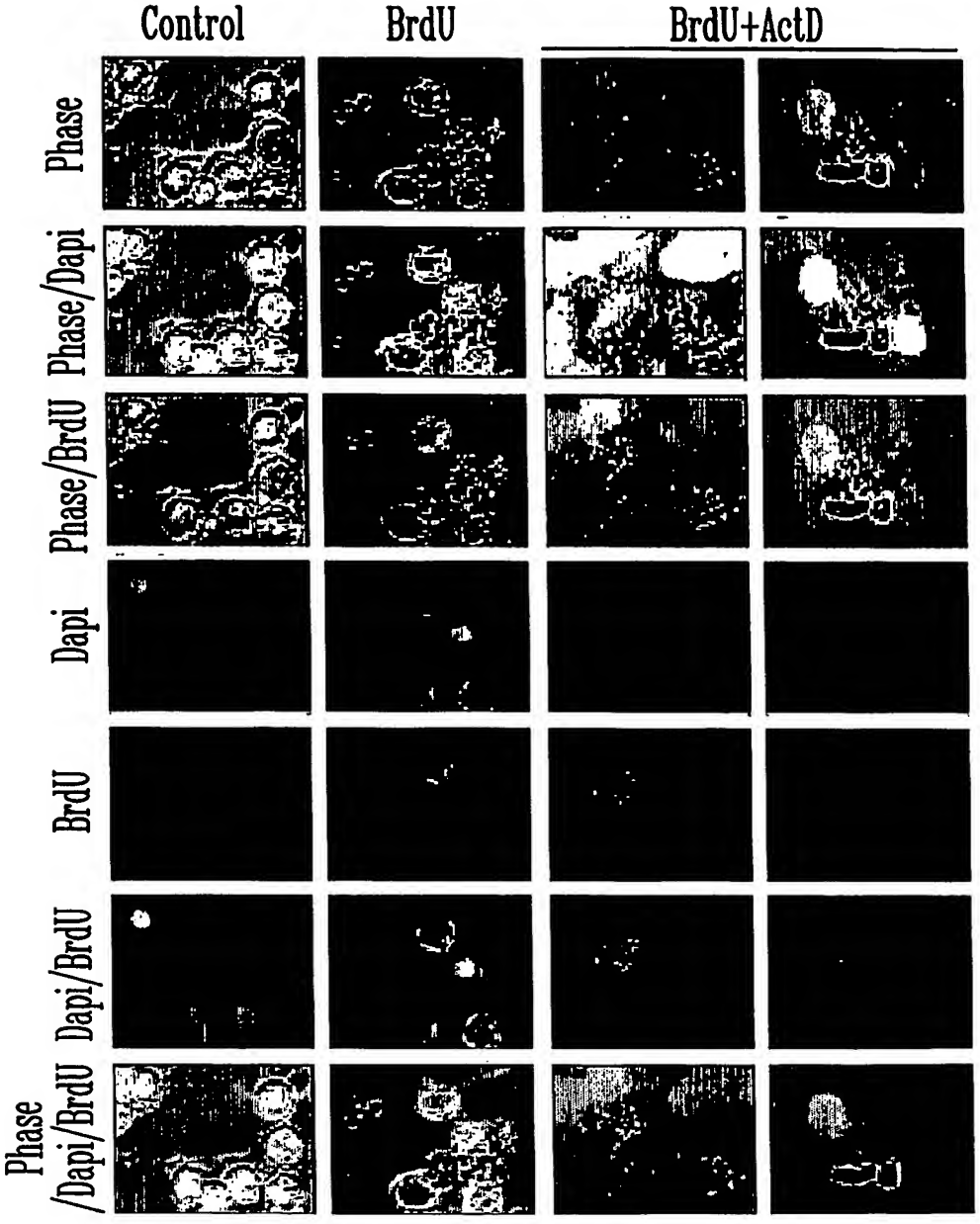

Control
BrdU
BrdU+ActD
Phase
Phase/Dapi
Phase/BrdU
Dapi
BrdU
Dapi/BrdU
Phase /Dapi/BrdU

FIG. 2B

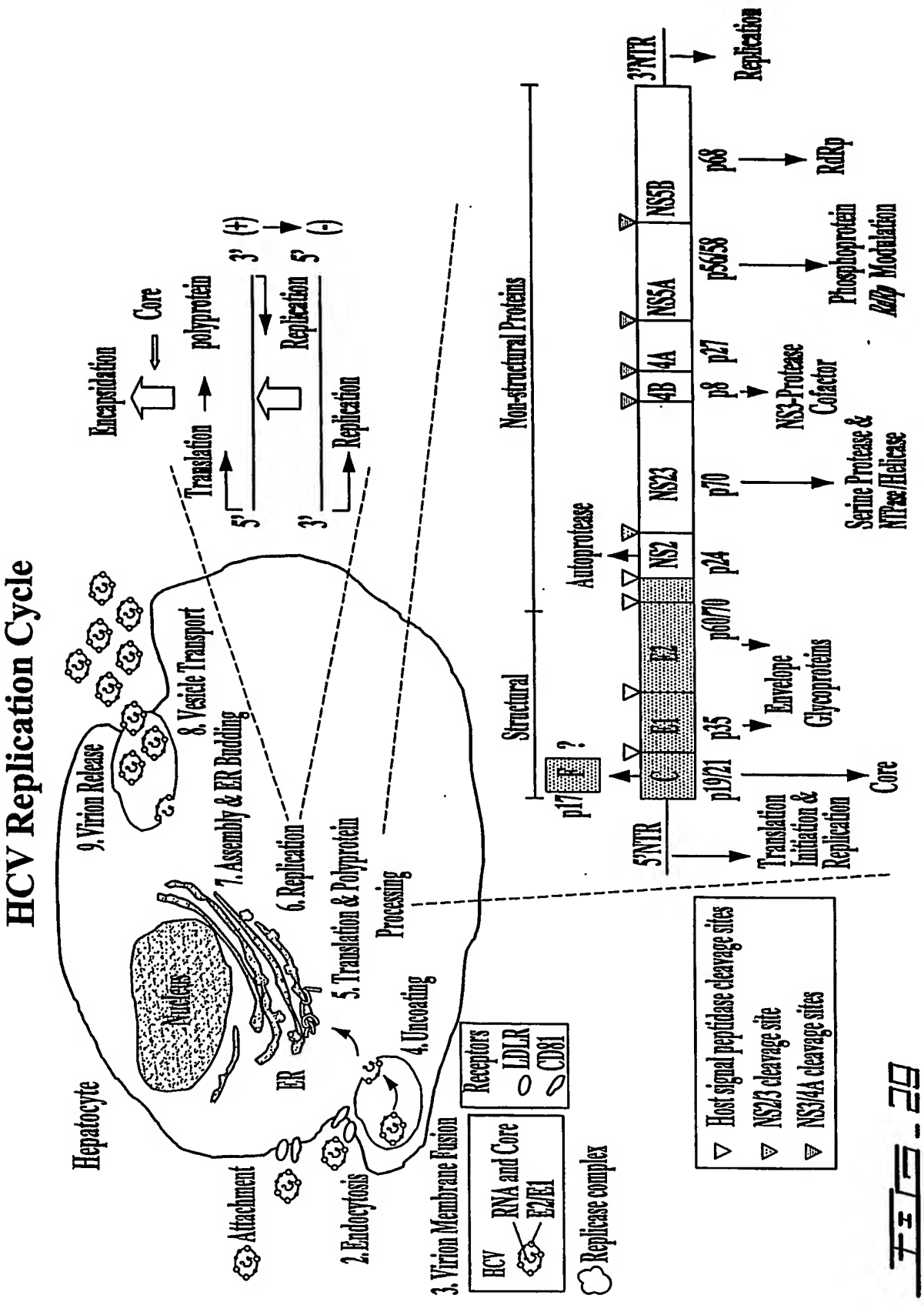

HCV Replication Cycle
Hepatocyte
Nucleus
ER
1. Attachment
2. Endocytosis
3. Virion Membrane Fusion
4. Uncoating
5. Translation & Polyprotein Processing
6. Replication
7. Assembly & ER Budding
8. Vesicle Transport
9. Virion Release
Encapsidation
Core
Translation
polyprotein
Replication
Replication
HCV
RNA and Core
E2/E1
Receptors
LDLR
CD81
Replicase complex
Structural
Non-structural Proteins
Autoprotease
5'NTR
C
E1
E2
p7
NS2
NS23
4B
4A
NS5A
NS5B
3'NTR
p17
p19/21
p35
p0070
p24
p70
p8
p27
p56/58
p68
Translation Initiation & Replication
Core
Envelope Glycoproteins
NS3-Protease Cofactor
Serine Protease & NTPase/Helicase
Phosphoprotein
Modulation
RdRp
Replication
Host signal peptidase cleavage sites
NS2/3 cleavage site
NS3/4A cleavage sites

FIG - 29

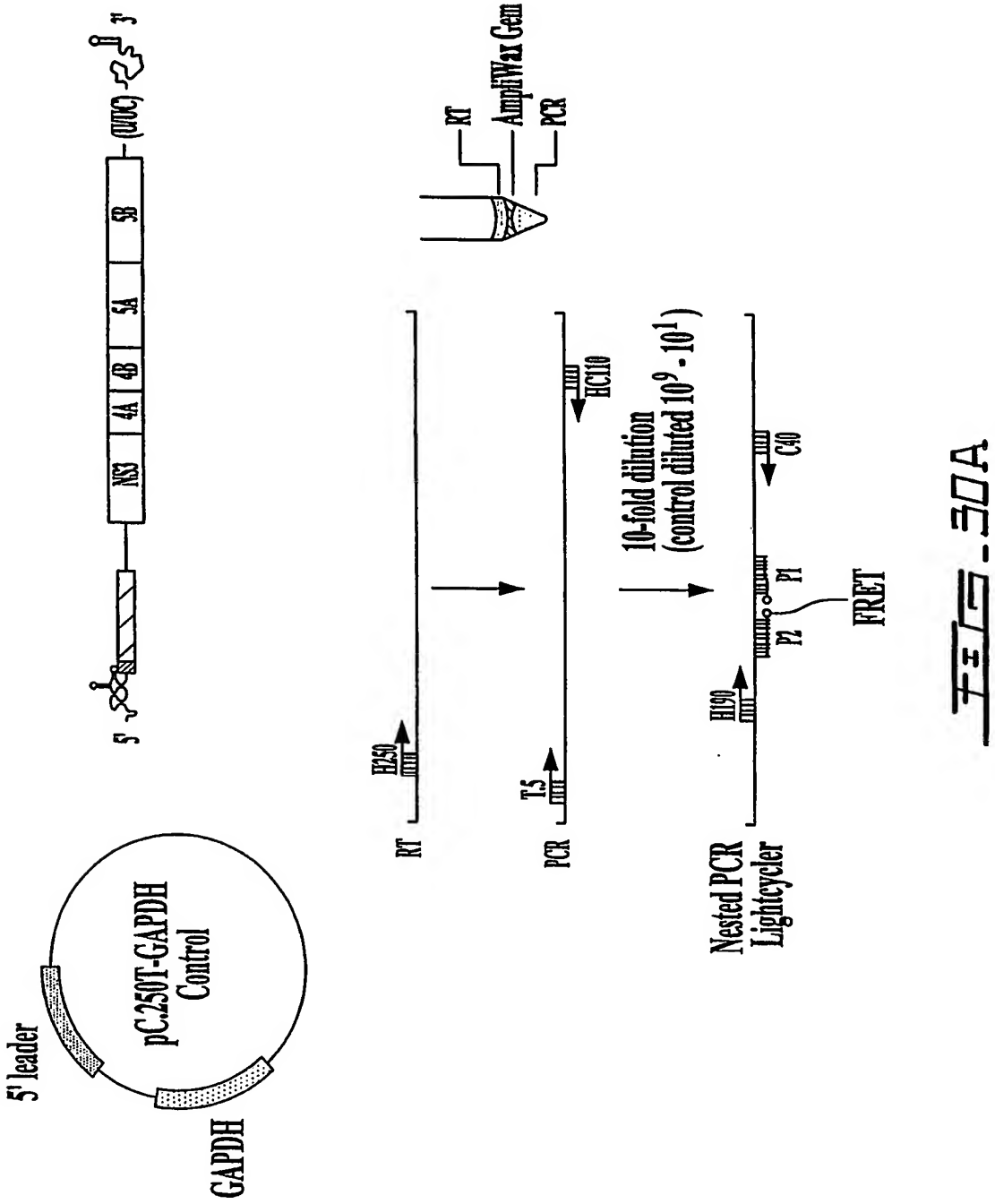

5' leader
pC250T-GAPDH
Control
GAPDH
5'
NS3
4A
4B
5A
5B
(AAA)n
3'
RT
AmpliWax Gem
PCR
H250
T5
HC110
RT
PCR
10-fold dilution
(control diluted 10^{-9} - 10^{-1})
H190
C40
P2
P1
FRET
Nested PCR
Lightcycler

FIG. 30A

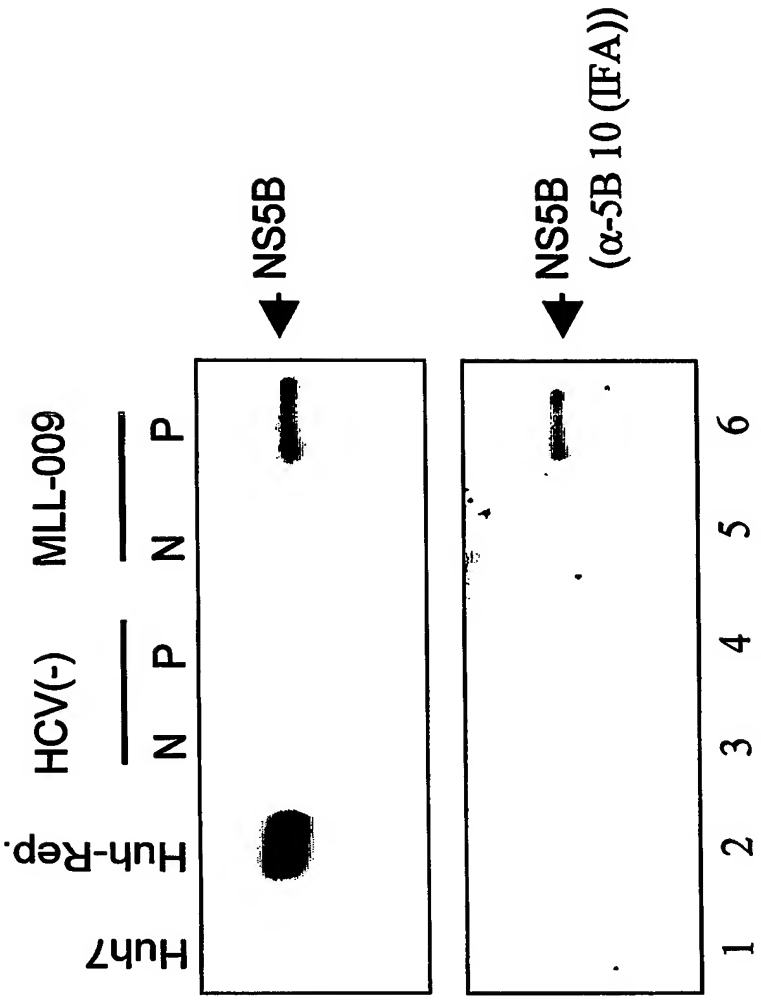


Fig. 30B

Fig. 31

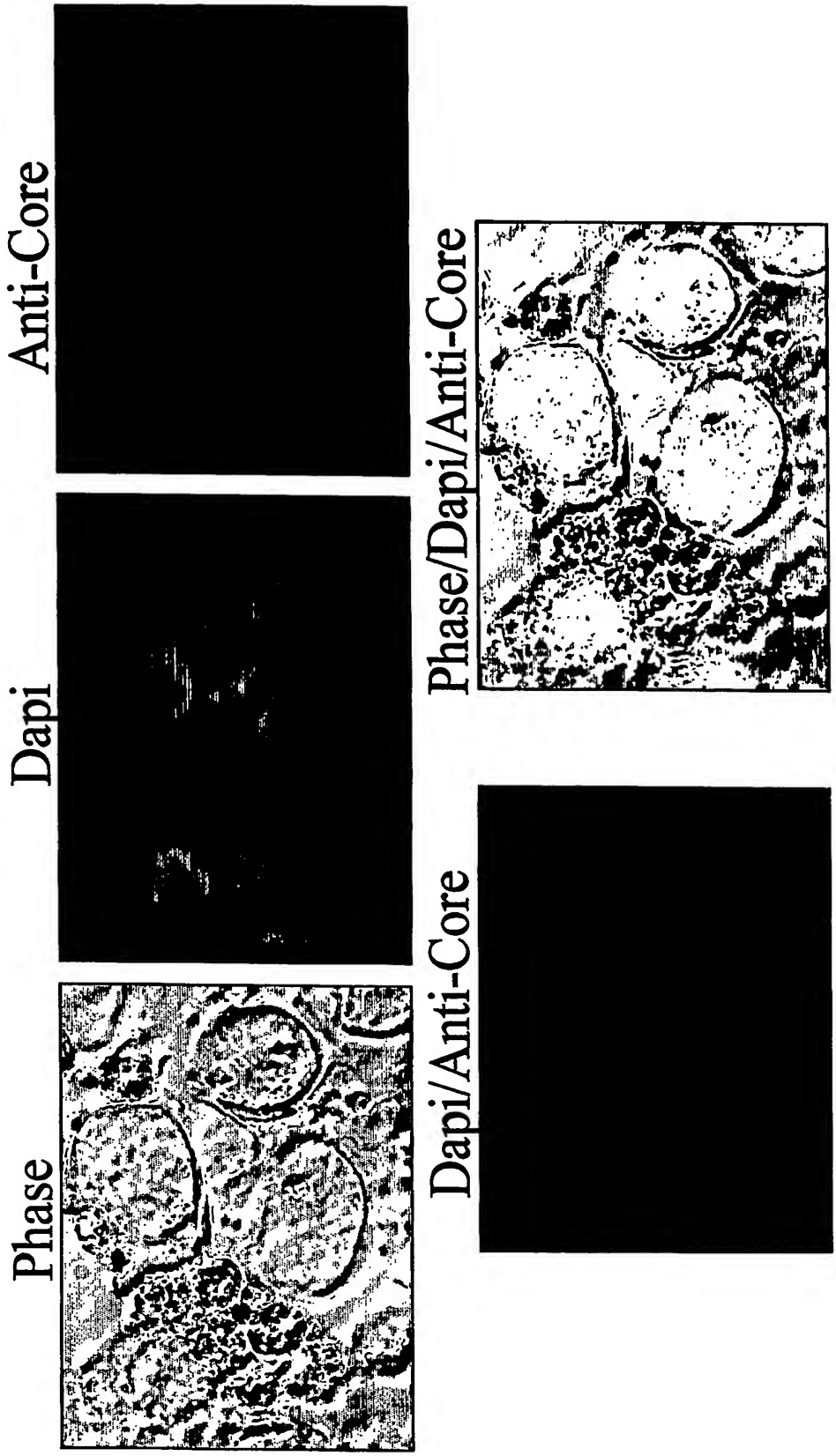


Fig. 32

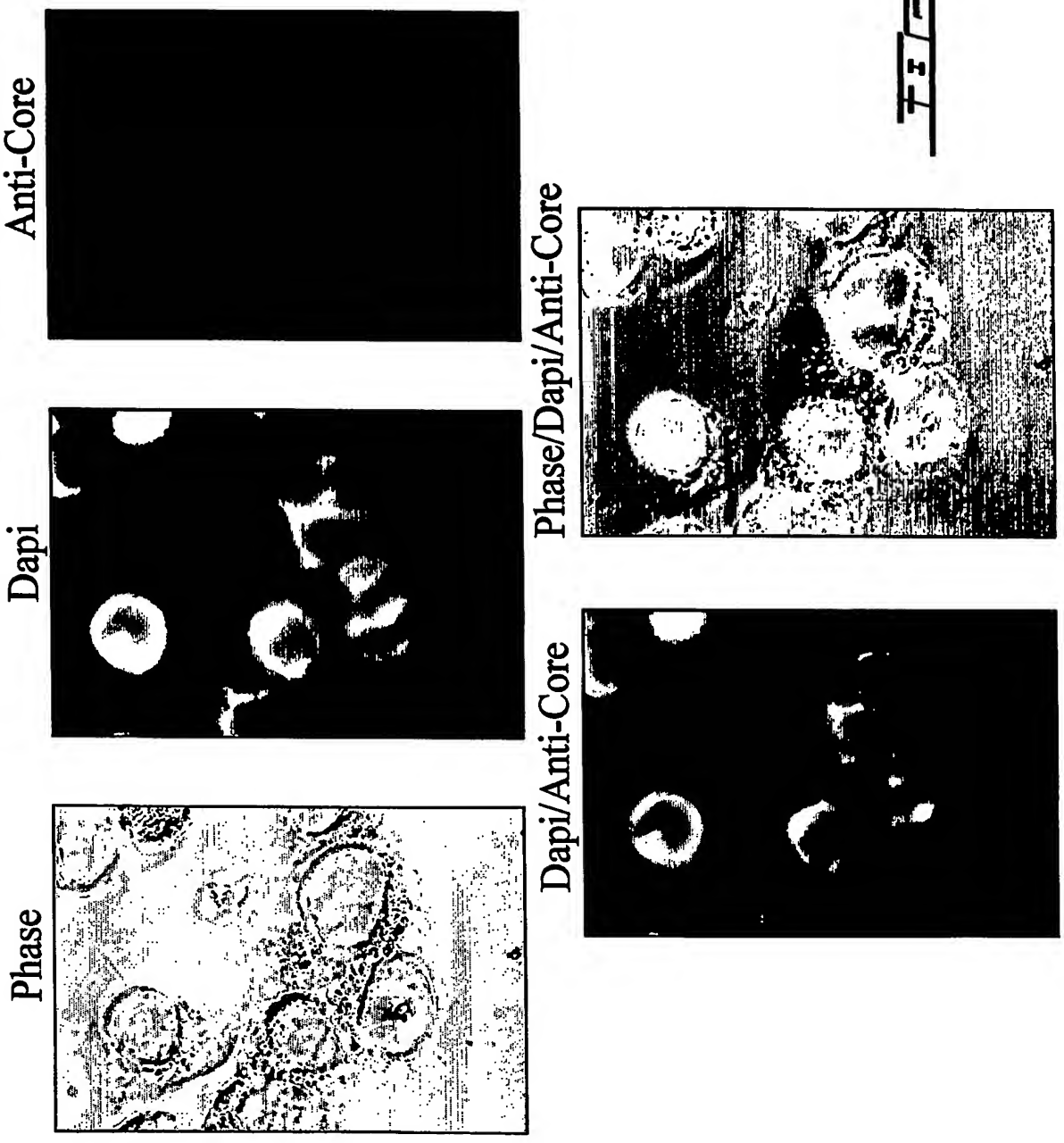


Fig. 33

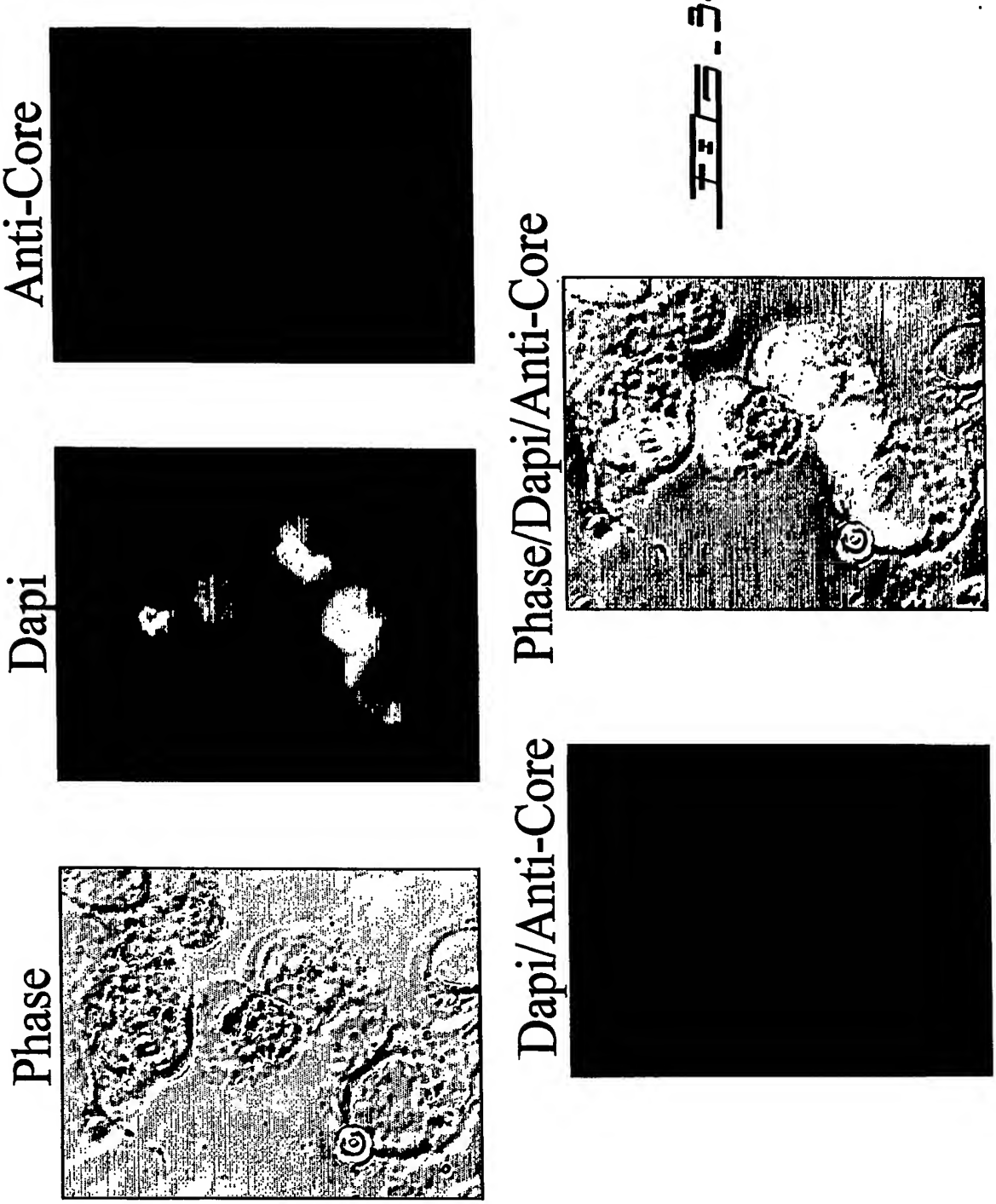


FIG. 34

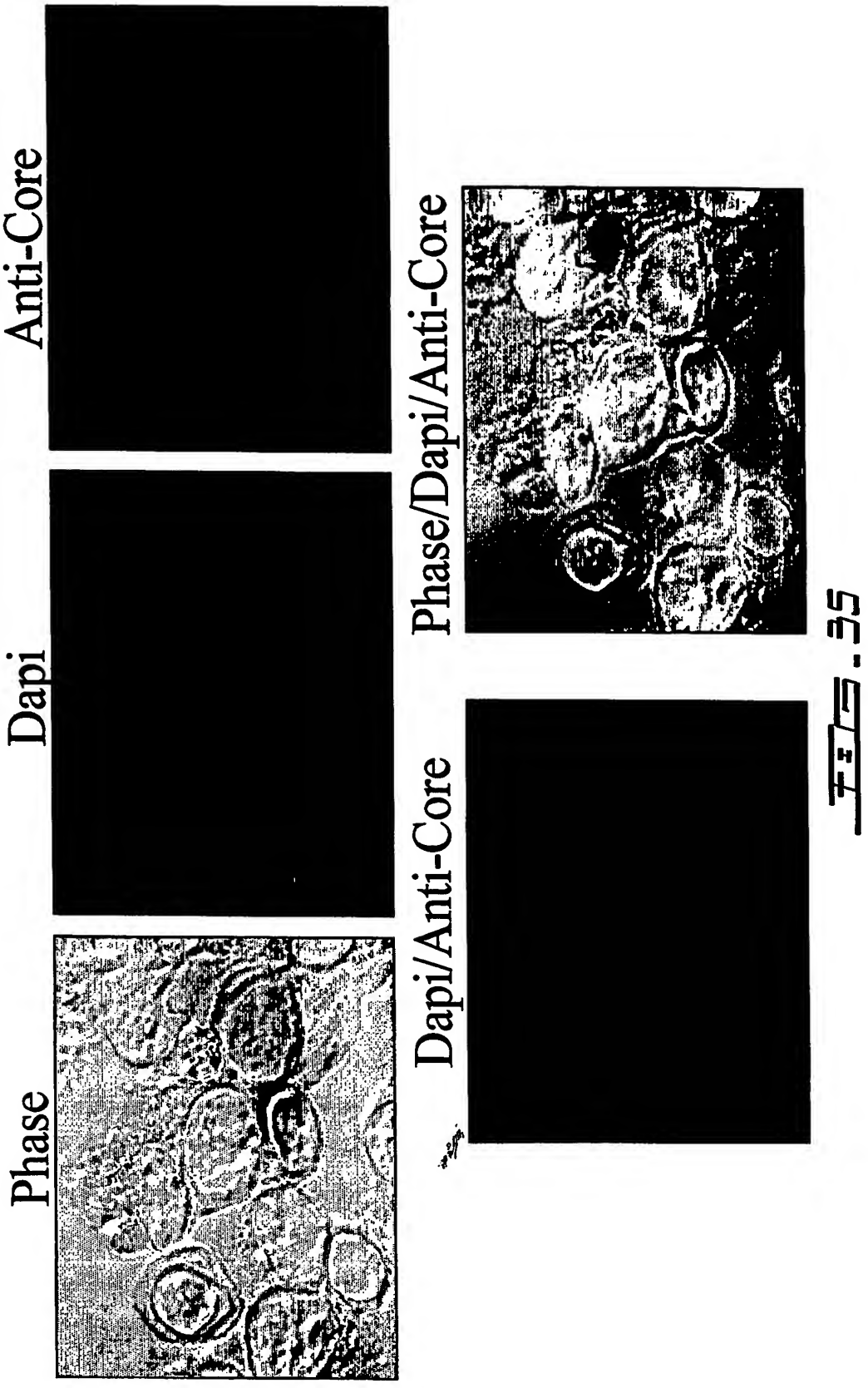


FIG. 35

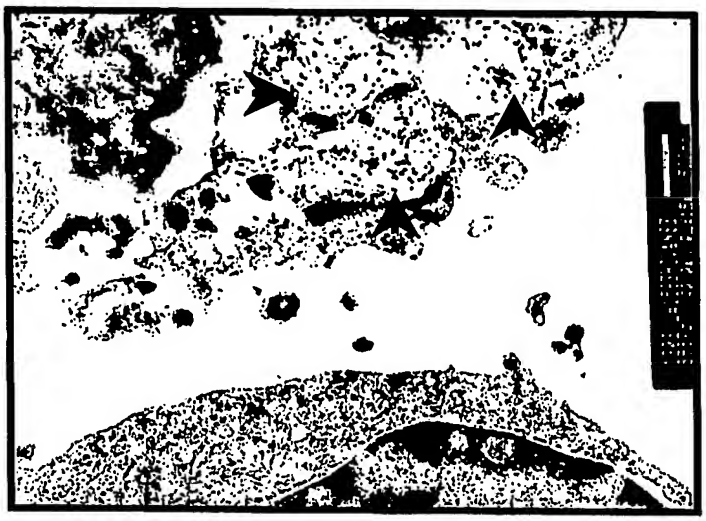


Fig. 36C

Fig. 36B

Fig. 36A

Fig. 37

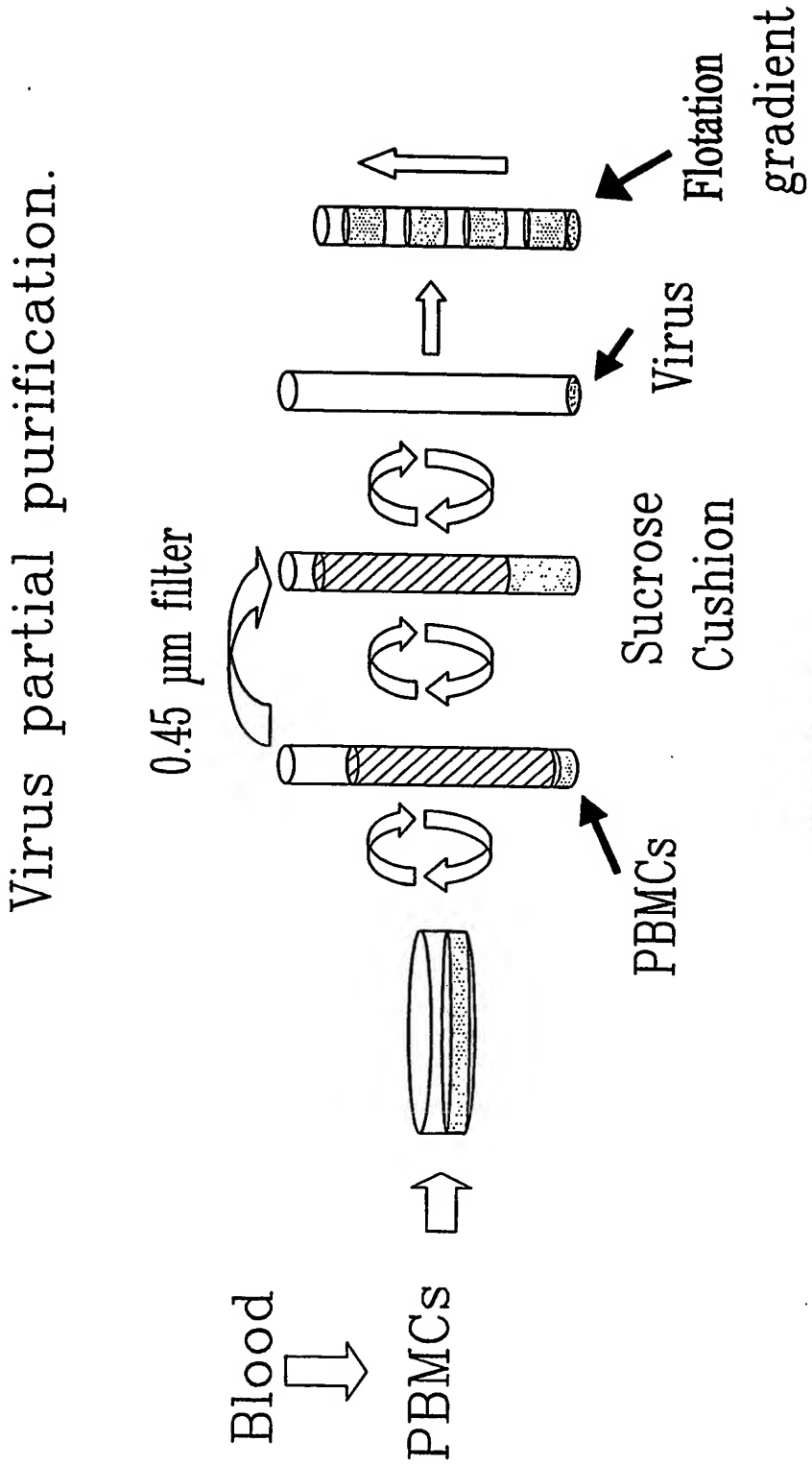

Virus partial purification.
Blood
PBMCs
0.45 µm filter
PBMCs
Sucrose Cushion
Virus
Flotation gradient

FIG. 3B

Density Range (g/ml)	Source	Reference
1.15-1.20	HCV-LP in VSV vector	J.Virol (2002) 76, 12325.
1.14-1.18	HCV-LP in insect cells	J. Virol (1998) 72, 3827.
1.12-1.17	Plasma chimps	J. Gen.Virol (1994) 75, 1755
1.09-1.21	Plasma chimps	J.Med.Virol (1991), 34, 206.
1.13-1.17	Plasma chimps	J.Virol (1993) 67, 1953
1.063-1.21	Serum infected donors	J Med Virol (2002) 68, 335
1.11-1.215	HCV(+) PBMCs	----------

FIG. 39

SUBSTITUTE SHEET (RULE 26)

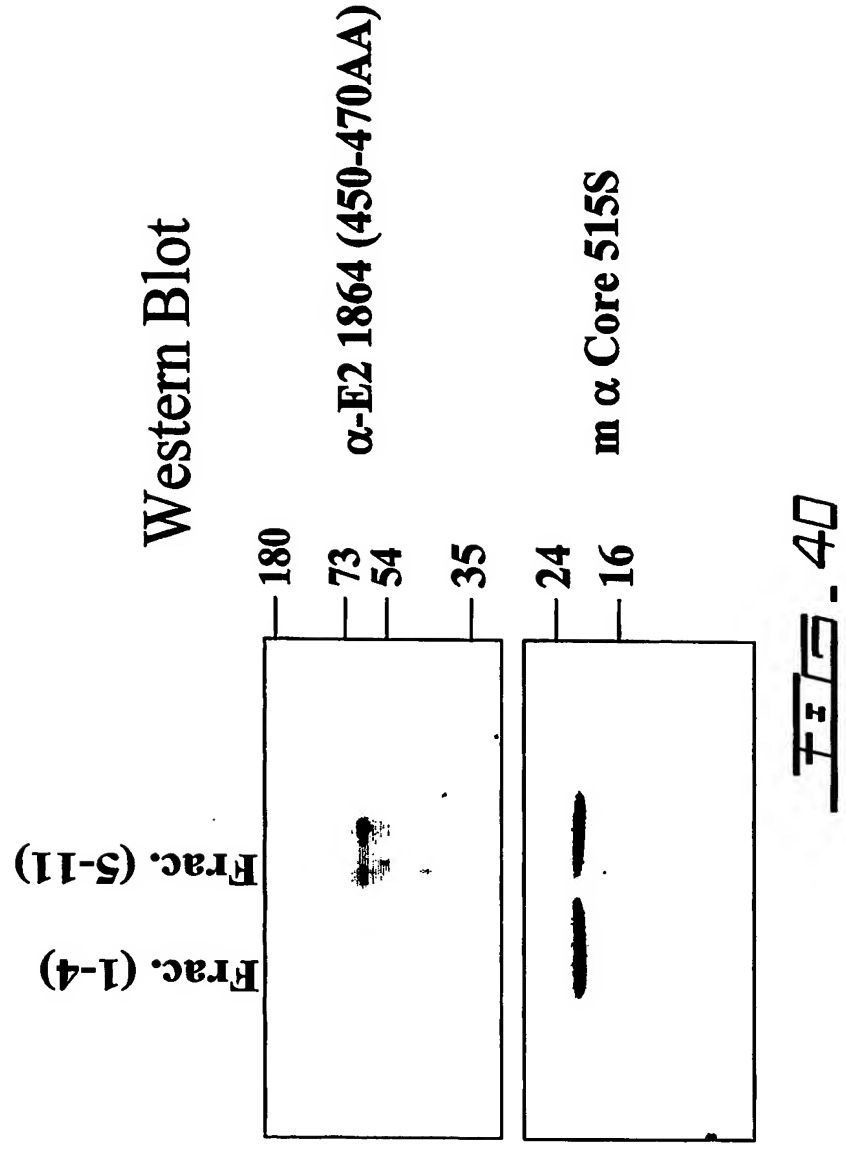

Western Blot
α-E2 1864 (450-470AA)
m α Core 515S
180
73
54
35
24
16
Frac. (5-11)
Frac. (1-4)

Fig. 4D

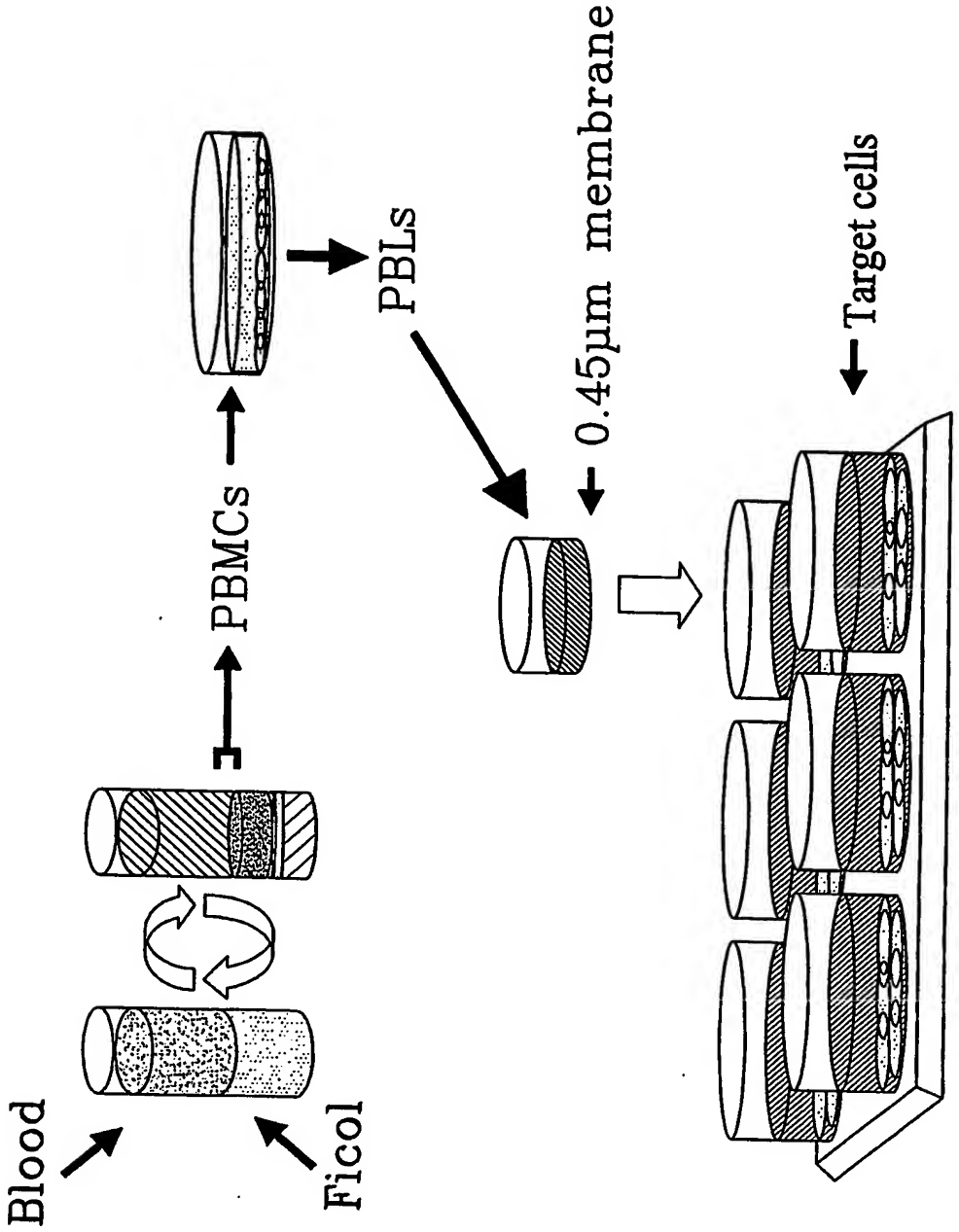


Fig. 41

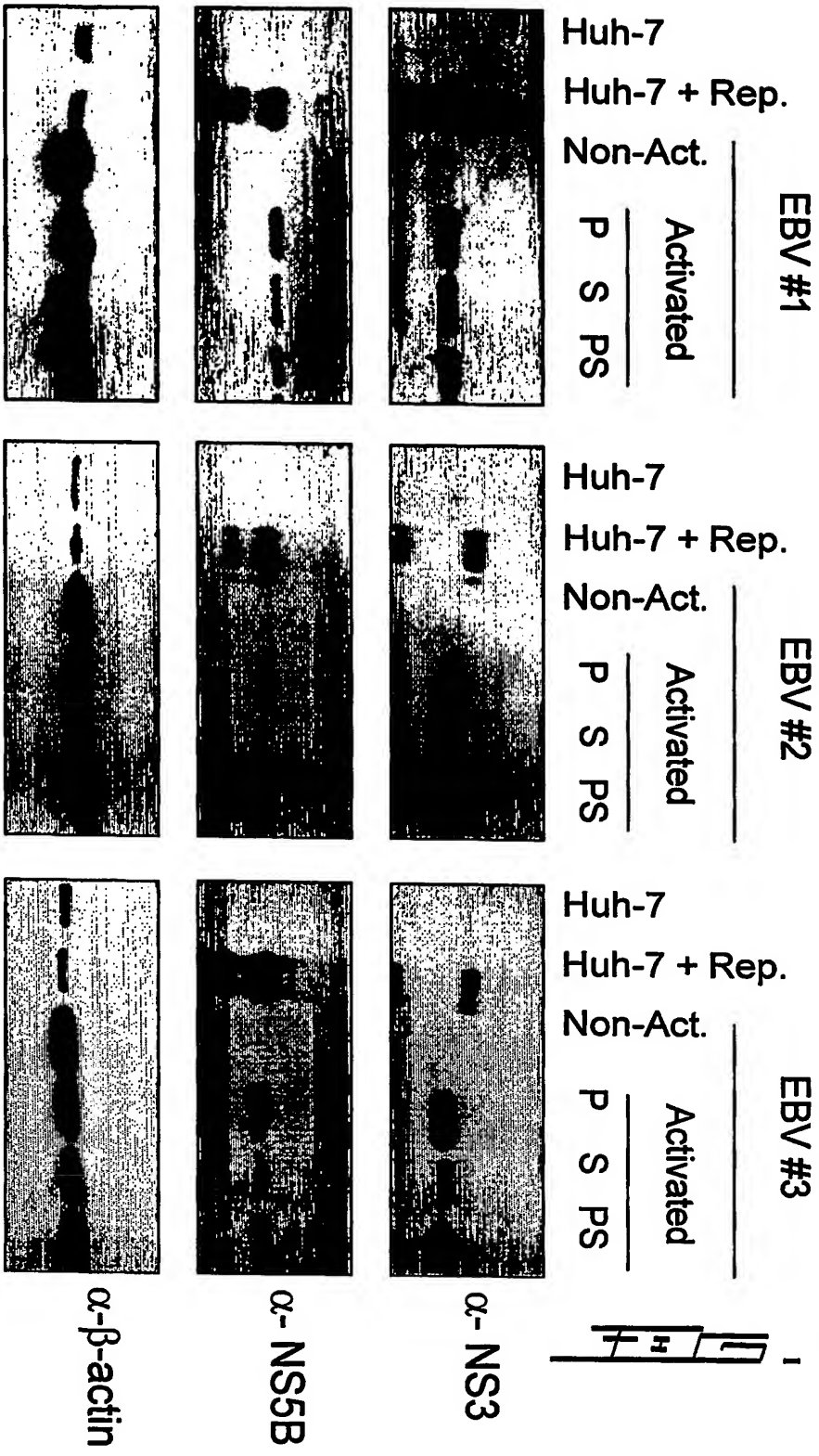

Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P
S
PS
EBV #1
Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P
S
PS
EBV #2
Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P
S
PS
EBV #3
α-β-actin
α- NS5B
α- NS3

FIG. 42A

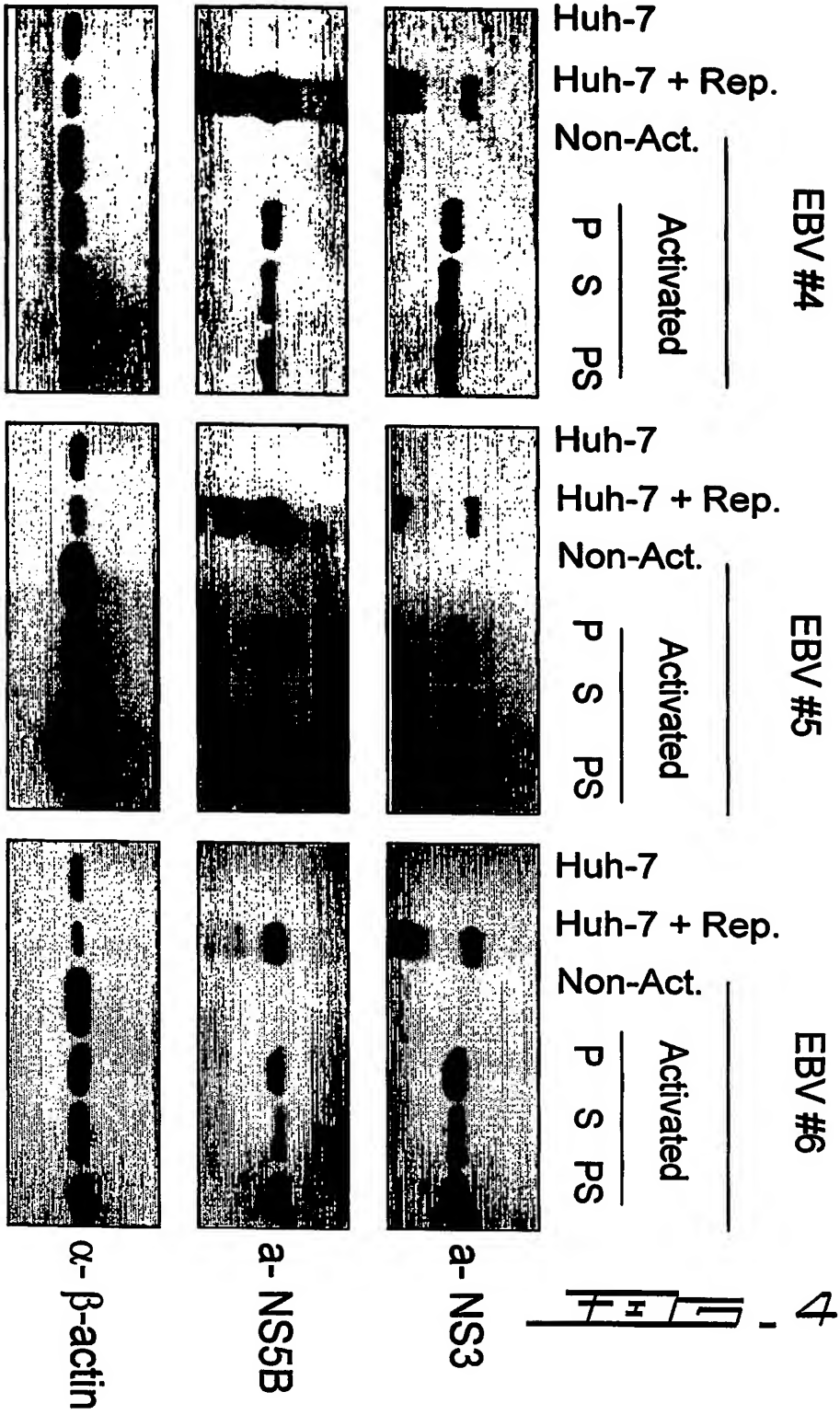

Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P S PS
EBV #4
Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P S PS
EBV #5
Huh-7
Huh-7 + Rep.
Non-Act.
Activated
P S PS
EBV #6
α- β-actin
a- NS5B
a- NS3

FIG - 42B

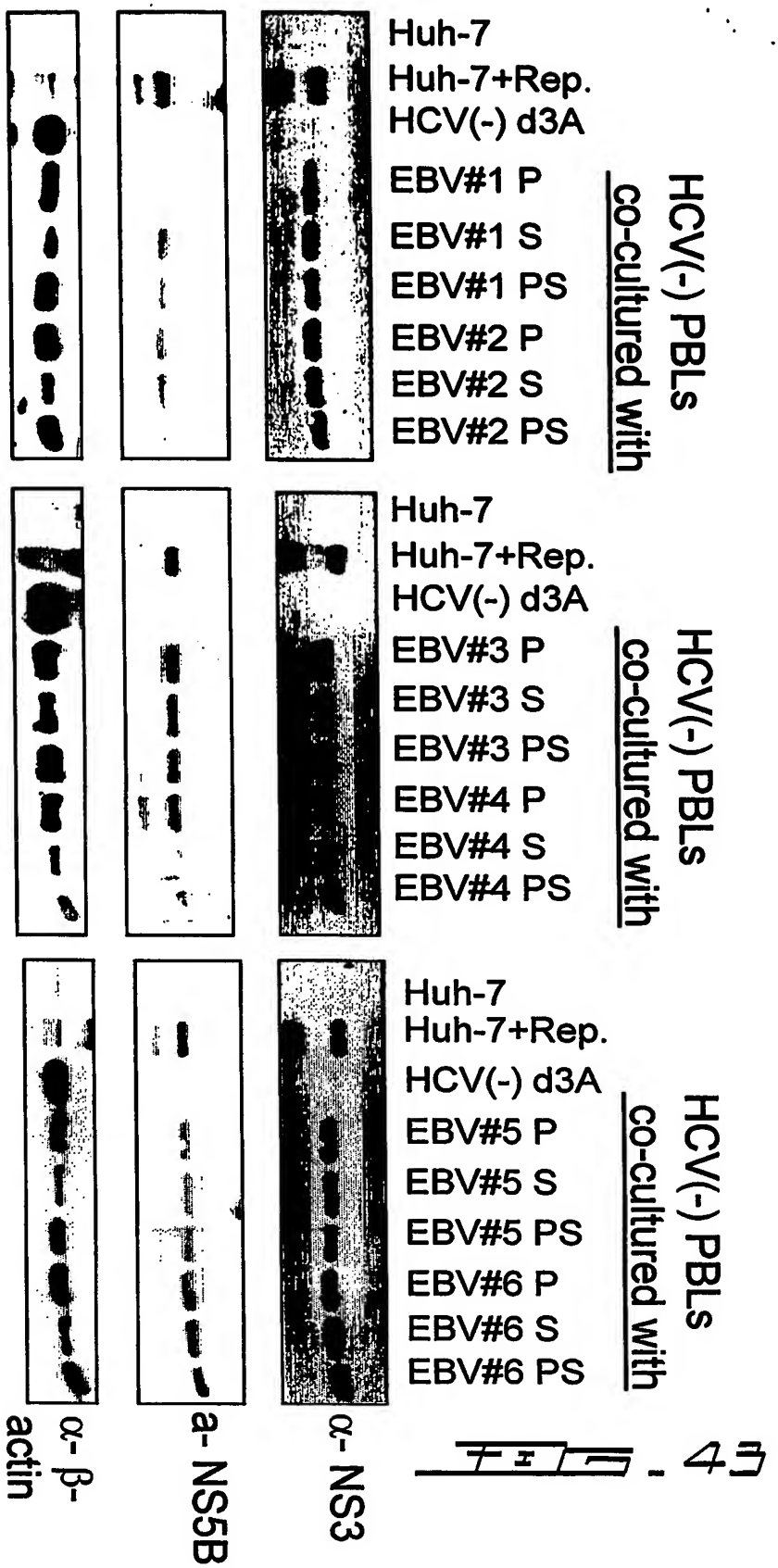

Huh-7
Huh-7+Rep.
HCV(-) d3A
EBV#1 P
EBV#1 S
EBV#1 PS
EBV#2 P
EBV#2 S
EBV#2 PS
HCV(-) PBLs co-cultured with
Huh-7
Huh-7+Rep.
HCV(-) d3A
EBV#3 P
EBV#3 S
EBV#3 PS
EBV#4 P
EBV#4 S
EBV#4 PS
HCV(-) PBLs co-cultured with
Huh-7
Huh-7+Rep.
HCV(-) d3A
EBV#5 P
EBV#5 S
EBV#5 PS
EBV#6 P
EBV#6 S
EBV#6 PS
HCV(-) PBLs co-cultured with
α- β-actin
a- NS5B
α- NS3

Fig. 4j

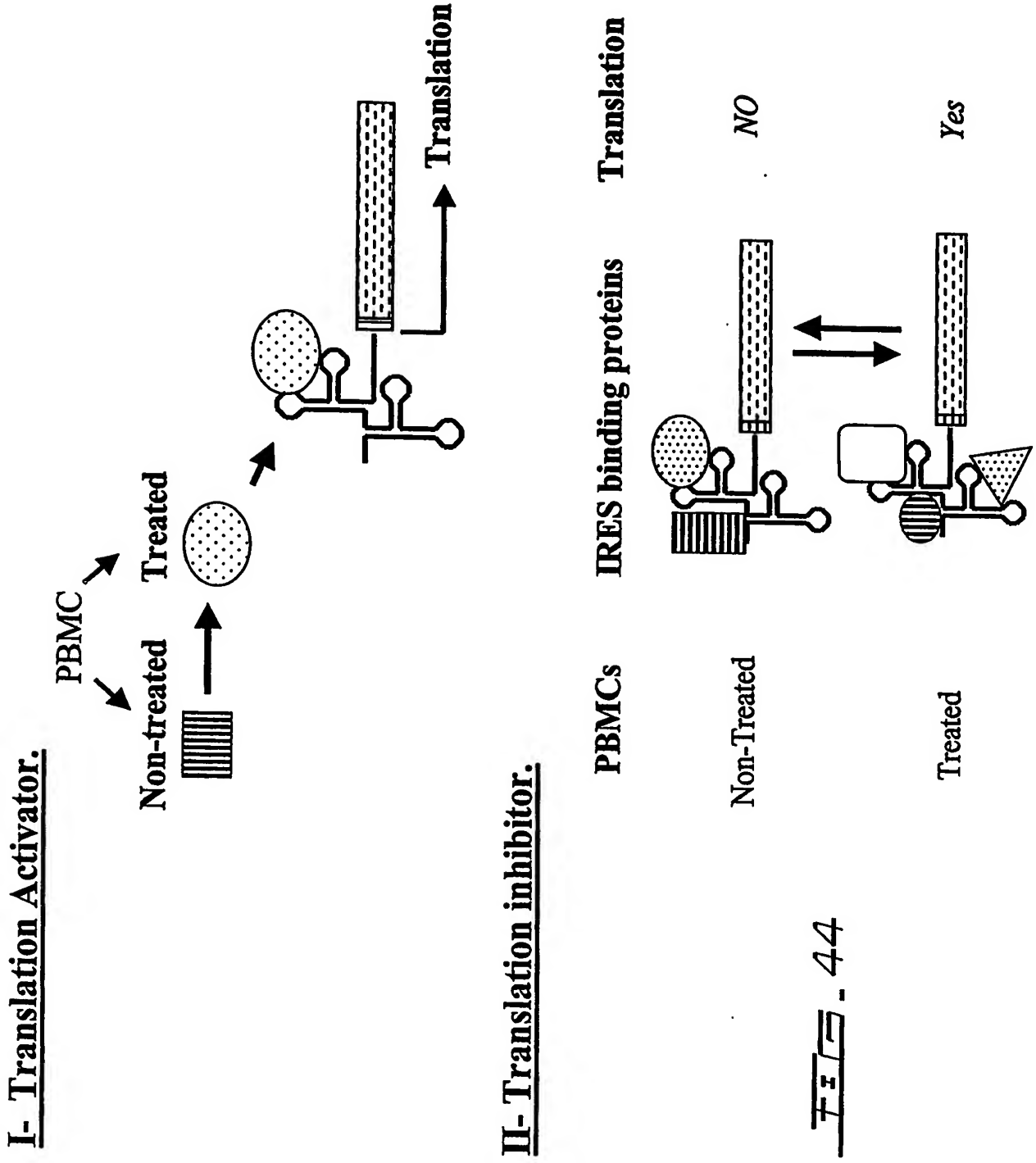
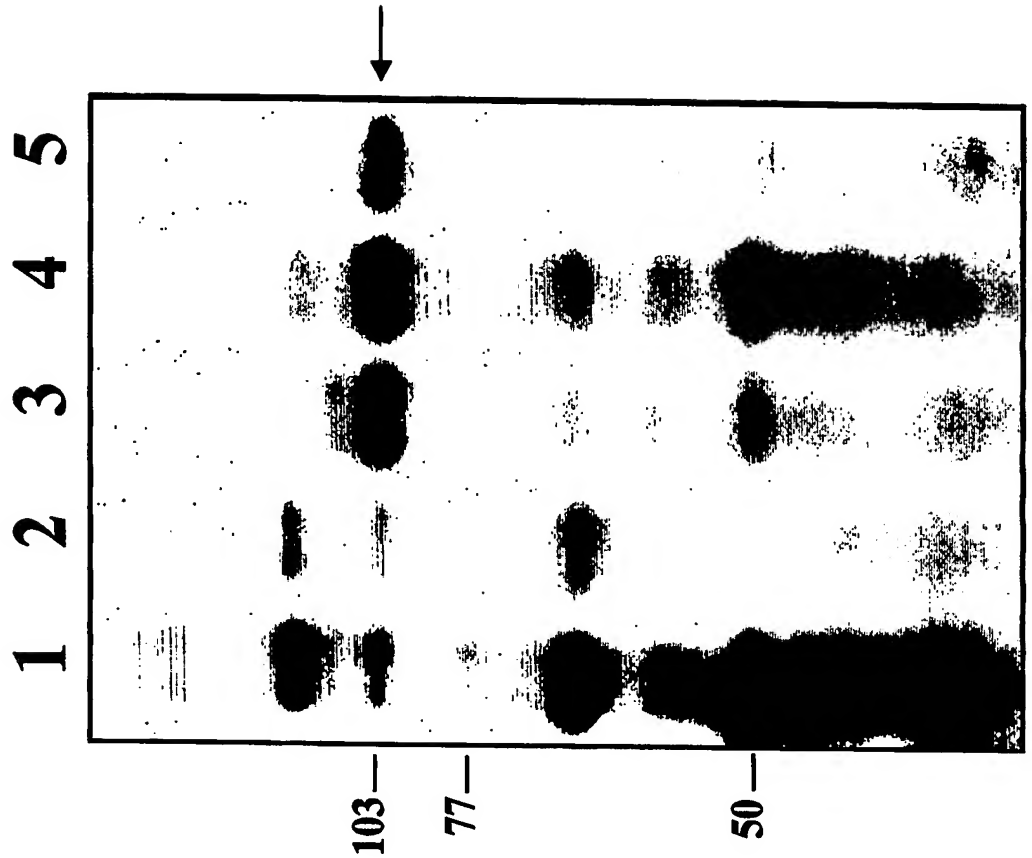
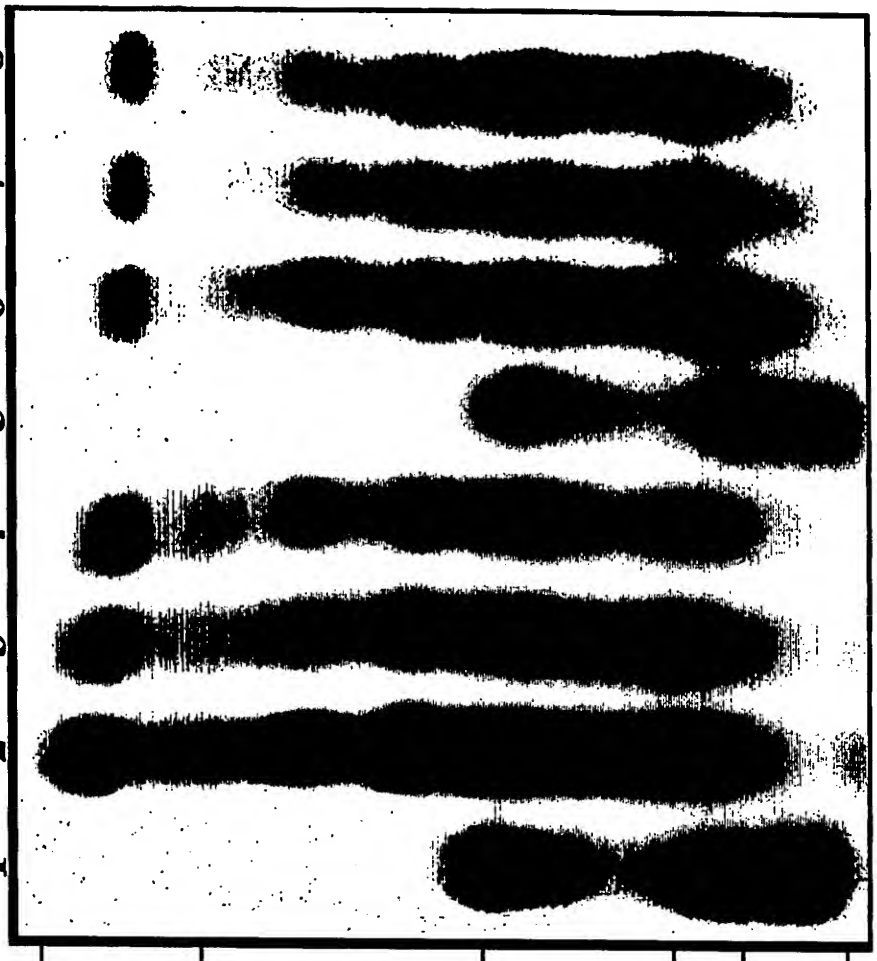

I- Translation Activator.
PBMC
Non-treated
Treated
Translation
II- Translation inhibitor.
PBMCs
IRES binding proteins
Translation
Non-Treated
NO
Treated
Yes

Fig. 44



Lane:
1- HeLa
2- HeLa + tRNA
3- HeLa + EMCV IRES
4- HeLa + polio IRES
5- HeLa + ODC IRES

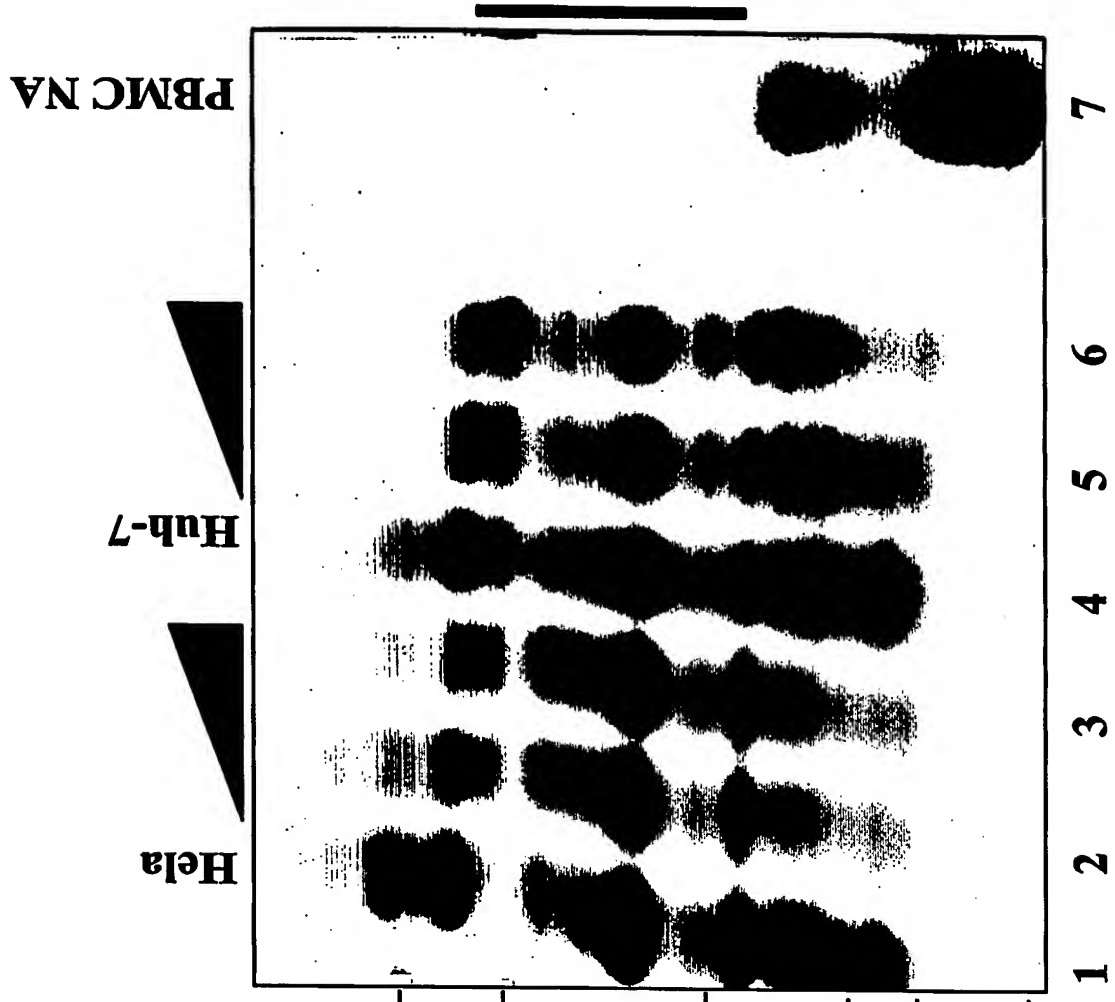
Fig. 45



Lane:

1- PBMCs NT
2- PBMCs treatment 1
3- PBMCs treatment 2
4- PBMCs treatment 2+DEVA
5- PBMCs NT + HIV
6-PBMCs treatment 1 +HIV
7- PBMCs treatment 2 +HIV
8- PBMCs treatment 2 +DEVA+HIV

FIG. 46

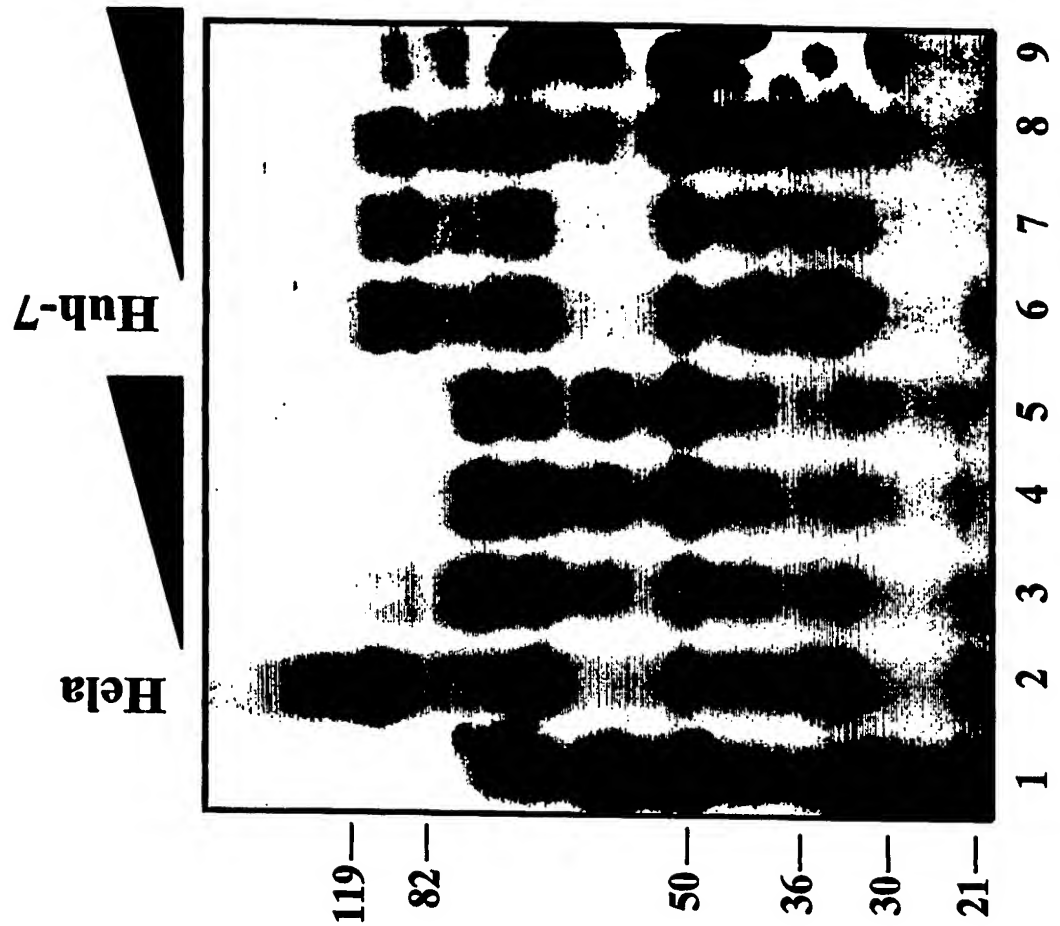


Lane:

1- HeLa (40 ug)
2- Hela + PBMCs NT(6 ug)
3- HeLa + PBMCs NT (12ug)
4- Huh-7 (38 ug)
5- Huh-7 + PBMCs NT (6ug)
6- Huh-7 + PBMCs NT (12ug)
7- PBMCs NT (12 ug)

PBMC are HCV(-)

Fig. 47



Lane:

1- HCV (-) PBMCs NA (20 ug)

2- HeLa (20 ug)

3- HeLa + HCV (-) PBMCs NT (5 ug)

4- HeLa + HCV (-) PBMCs NT (10 ug)

5- HeLa + HCV (-) PBMCs NT (20 ug)

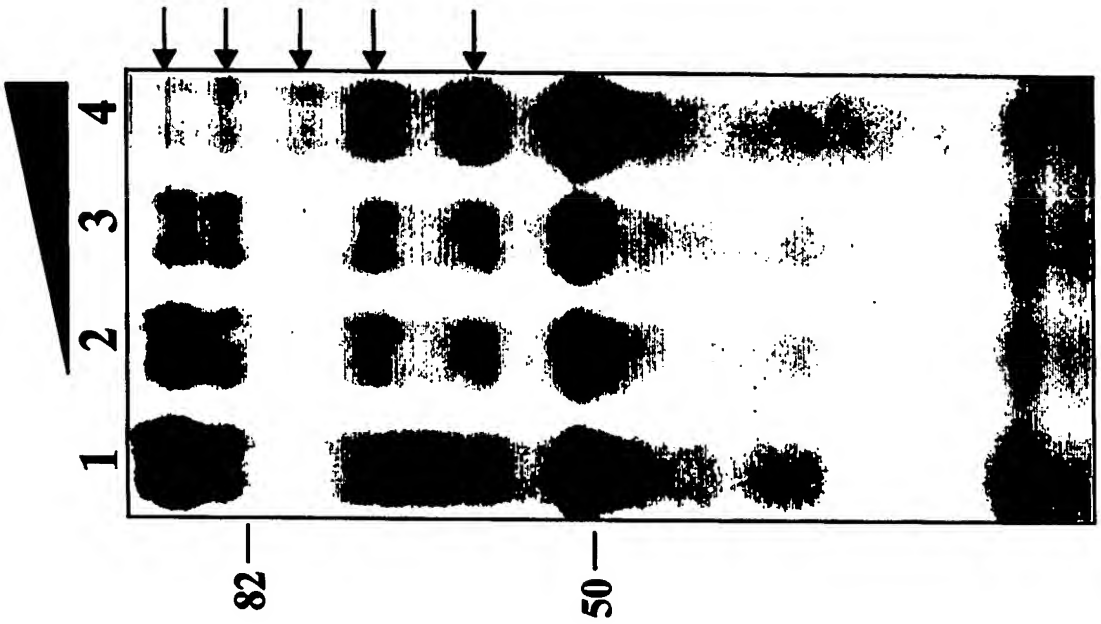
6- Huh-7 (20 ug)

7- Huh-7 + HCV (-) PBMCs NT (5 ug)

8- Huh-7 + HCV (-) PBMCs NT (10 ug)

9- Huh-7 + HCV (-) PBMCs NT (20 ug)

FIG. 4B



Lane:

1- Huh-7 (20ug)
2- Huh-7 + HCV (-) PBMCs NT (5ug)
3- Huh-7 + HCV (-) PBMCs NT (10ug)
4- Huh-7 + HCV (-) PBMCs NT (20ug)

Fig. 49

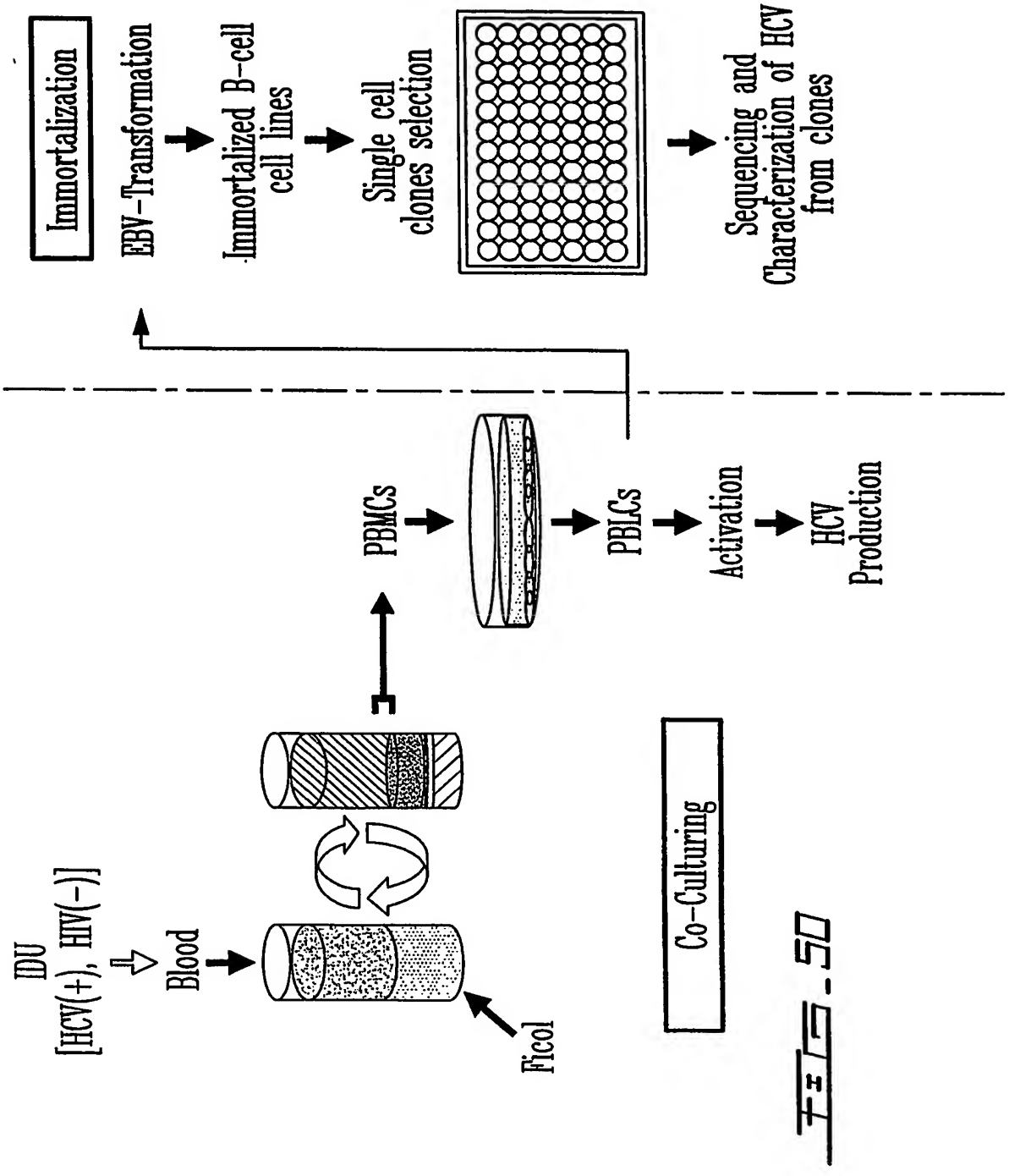

IDU
[HCV(+), HIV(-)]
Blood
Ficol
PBMCs
PBLCs
Activation
HCV
Production
Co-Culturing
Immortalization
EBV-Transformation
Immortalized B-cell
cell lines
Single cell
clones selection
Sequencing and
Characterization of HCV
from clones

FIG. 5D

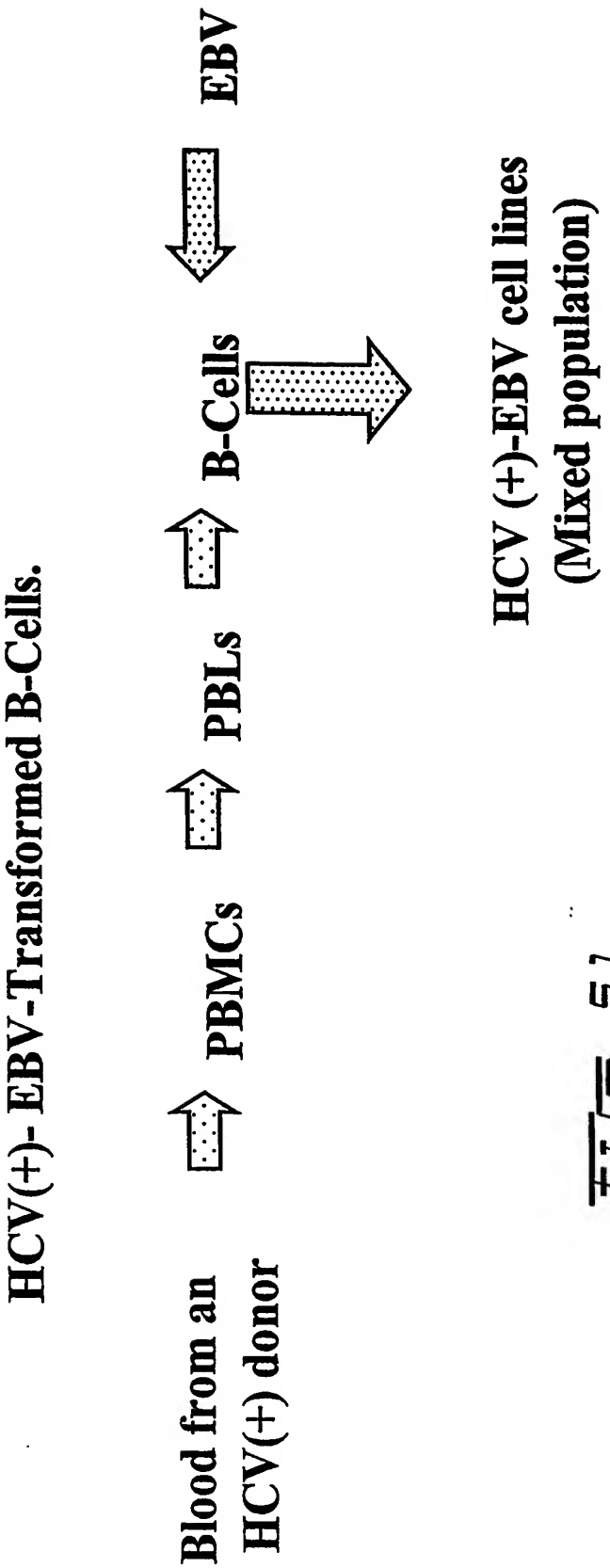

HCV(+)- EBV-Transformed B-Cells.
Blood from an HCV(+) donor
PBMCs
PBLs
B-Cells
EBV
HCV (+)-EBV cell lines (Mixed population)

Fig. 51

HCV RNA is detected in mixed population of EBV-transformed B-cells

HCV (+) Strand RNA

	Non-Stimulated cells	Stimulated cells
Cell line	RNA Copies /10^6 cells	RNA Copies /10^6 cells
EBV-1	4.66×10^5	2.33×10^6
EBV-2	2.77×10^5	7.91×10^4
EBV-3	3.96×10^6	4.02×10^5
EBV-4	2.03×10^6	1.57×10^6
EBV-6	1.41×10^6	4.32×10^5
EBV-HCV (-)	0	0

GAPDH mRNA

	Non-Stimulated cells	Stimulated cells
Cell line	RNA Copies /10^6 cells	RNA Copies /10^6 cells
EBV-1	2.23×10^8	2.19×10^8
EBV-2	8.73×10^8	2.25×10^8
EBV-3	1.83×10^9	1.77×10^9
EBV-4	5.48×10^8	3.79×10^8
EBV-6	1.26×10^9	9.42×10^8
EBV-HCV (-)	9.27×10^7	3.62×10^8

FIG - 52

SUBSTITUTE SHEET (RULE 26)

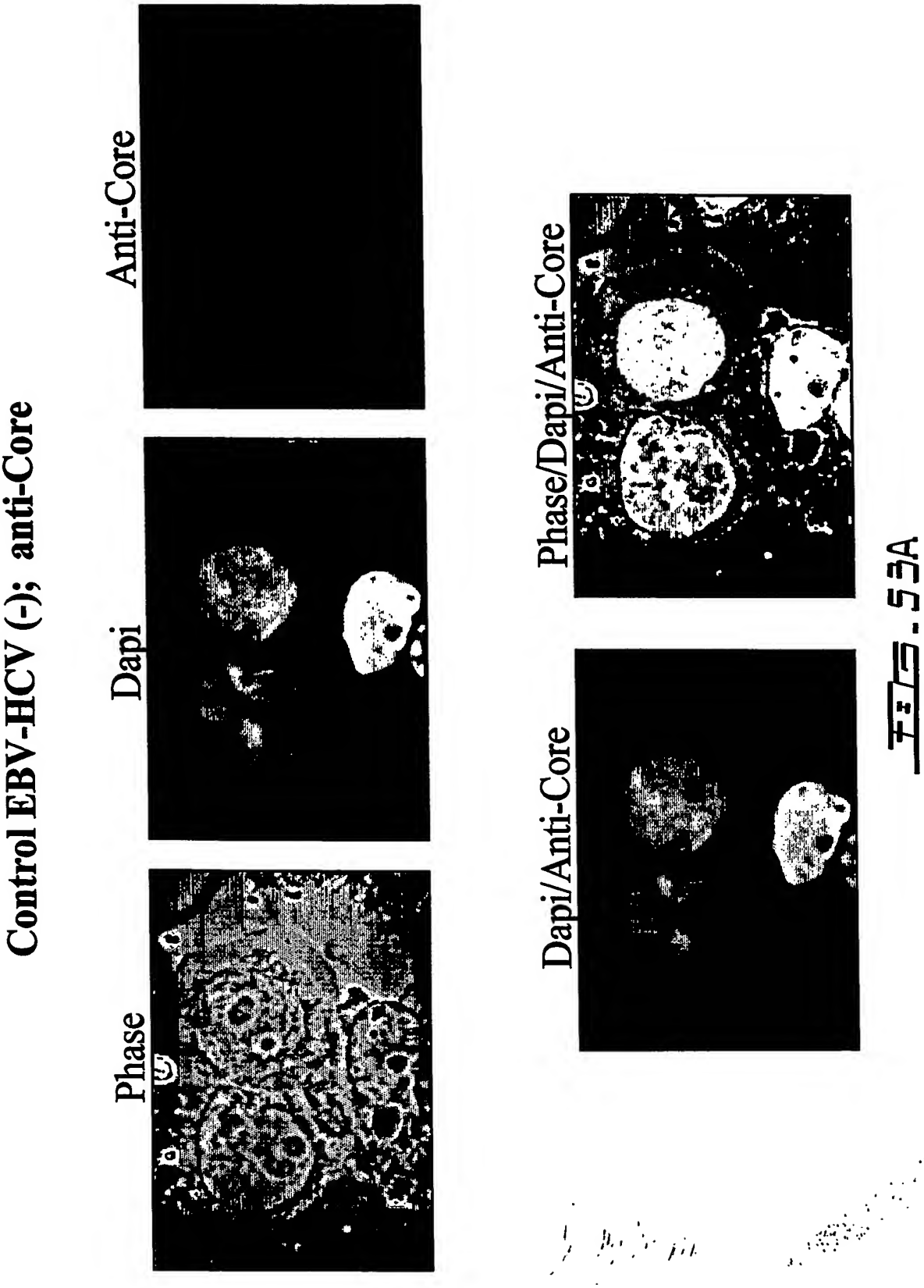

Control EBV-HCV (-); anti-Core
Phase
Dapi
Anti-Core
Dapi/Anti-Core
Phase/Dapi/Anti-Core

FIG. 53A

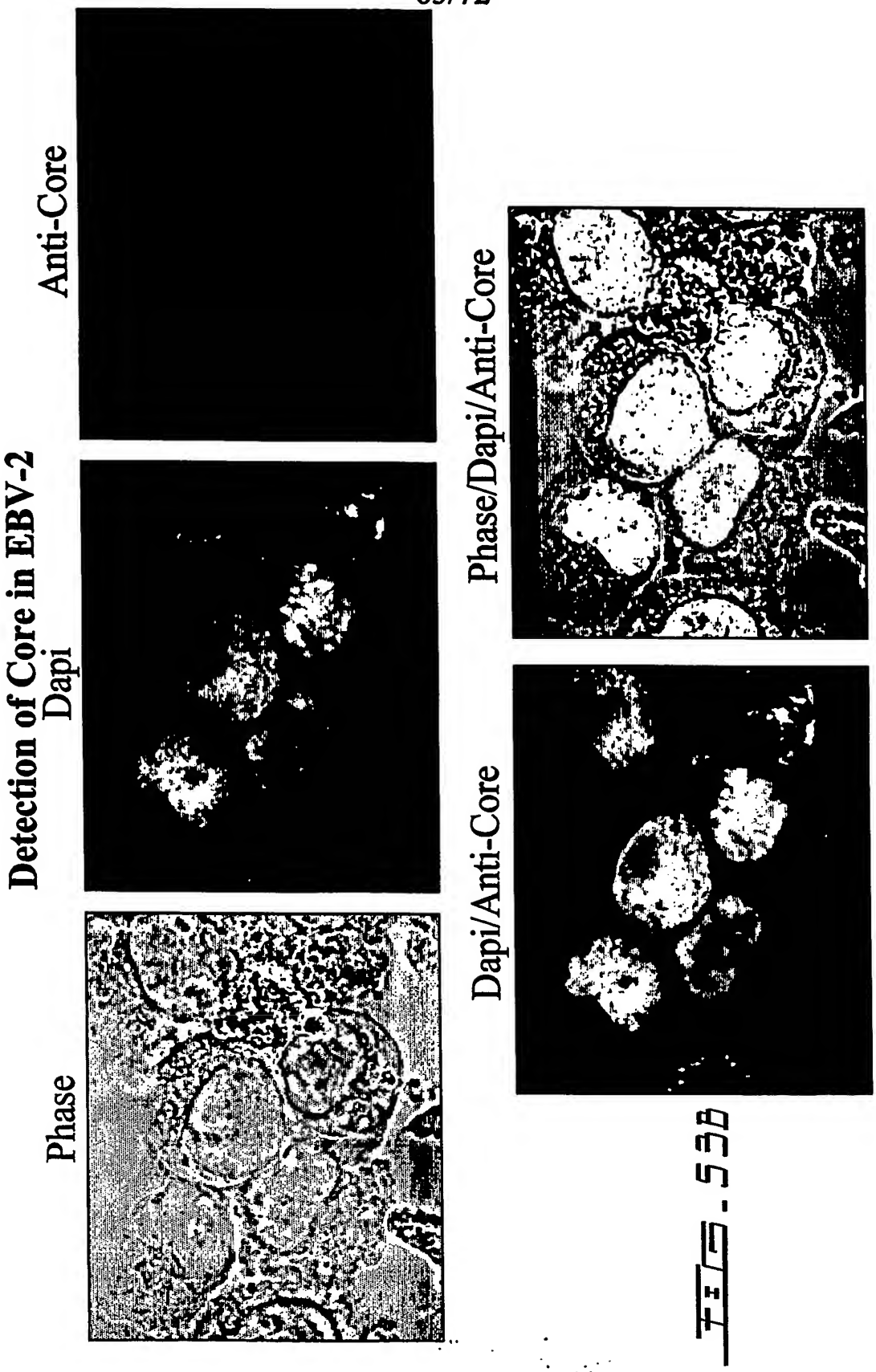

Detection of Core in EBV-2
Phase
Dapi
Anti-Core
Dapi/Anti-Core
Phase/Dapi/Anti-Core

FIG. 53B

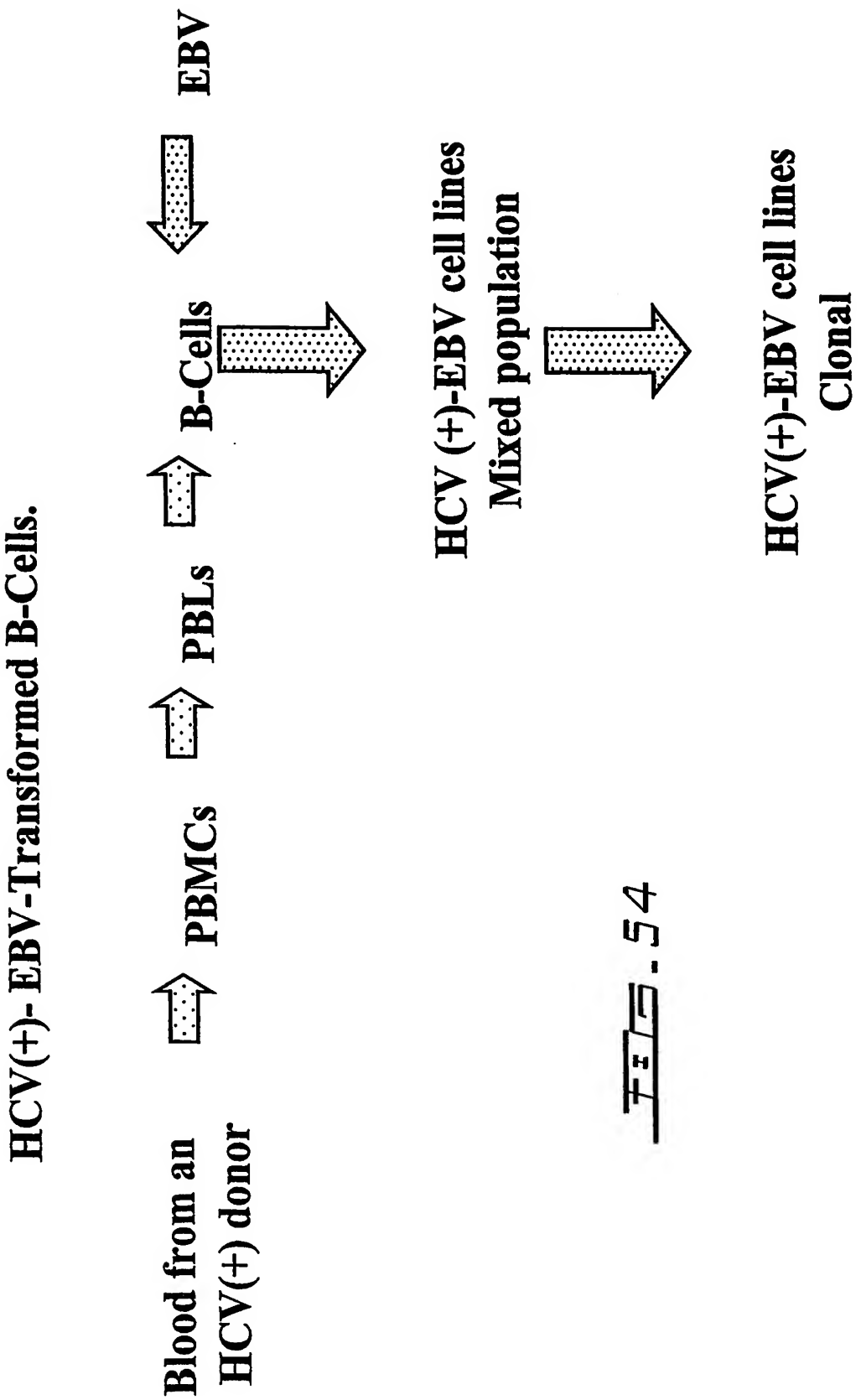

HCV(+)- EBV-Transformed B-Cells.
Blood from an HCV(+) donor
PBMCs
PBLs
B-Cells
EBV
HCV (+)-EBV cell lines Mixed population
HCV(+)-EBV cell lines Clonal

Fig. 54

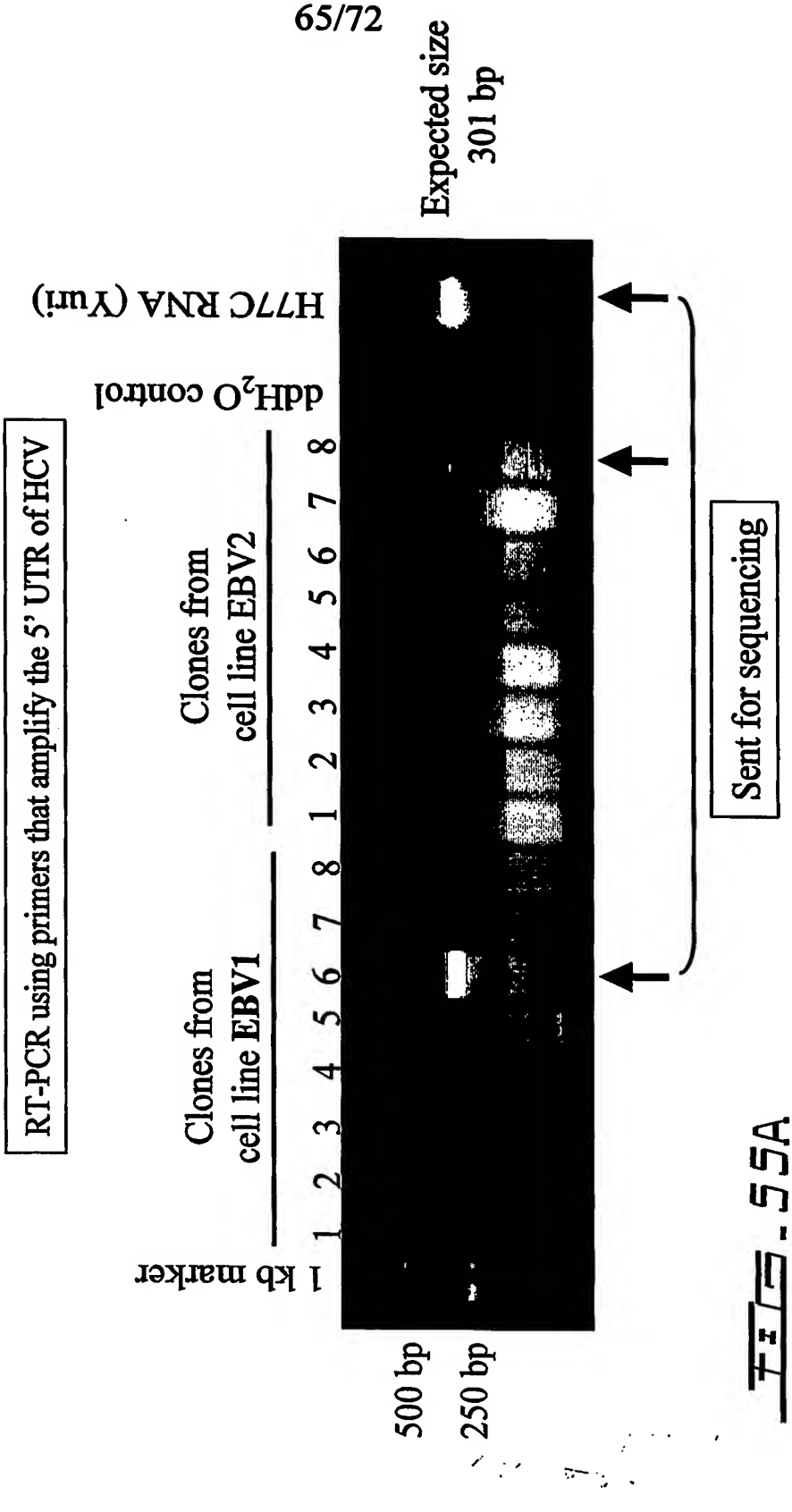

RT-PCR using primers that amplify the 5' UTR of HCV
1 kb marker
Clones from cell line EBV1
1 2 3 4 5 6 7 8
Clones from cell line EBV2
1 2 3 4 5 6 7 8
ddH2O control
H77C RNA (Yuri)
Expected size 301 bp
Sent for sequencing
500 bp
250 bp

FIG. 55A

Alignment: H77C (RT-PCR positive control) sequence (top)/
EBV1 clone 6 sequence (bottom)

CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGT
CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGT

TAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTC
TAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTC

G
TGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATAA
TGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATTA

ACCCGCTCACATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAG
ACCCGCTCA –ATGCCTGGAGATTTGGGCGTGCCCCCGCGAGACTGCTAGCCGAGTAG

TGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGT
TGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGT

FIG. 55B

Blue: sequence from virus in the serum (MLL-005).

Alignment: H77C (RT-PCR positive control) sequence (top)/ EBV2 clone 8 sequence (bottom).

CCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACC
CCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACC

GGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGG
GGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGG

ATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAG
ATAAA*T*CCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAG

ACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTAC
ACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTAC

TGCCTGATAGGGTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGAC
TGCCTGATAGGGTGCTTGCGAGTGC*T*CCGGGAGGTCTCGTAGAC

CGTGCA
CGTGCA

FIG. 55C

SUBSTITUTE SHEET (RULE 26)

EBV1 Clone 3 (1.3*)
EBV1 Clone 4 (1.4)
EBV1 Clone 5 (1.5)
EBV1 Clone 6 (1.6)
EBV1 Clone 7 (1.7)
EBV2 Clone 2 (2.2*)
EBV2 Clone 4 (2.4*)
EBV9 Clone 2 (9.2*)
EBV9 Clone 4 (9.4*)
- A B C D
H77C RNA (Yuri)
H77C RNA
ddH_2O control
sequencing
- = clone alone (not diluted with other cells)
A= diluted 1:10 with MT4 cell line (HTLV1 transformed T cells)
B= diluted 1:10 with BJAB cell line (ATCC non-EBV transformed B cells)
C= diluted 1:10 with HLA 006 cell line (EBV transformed HCV- PBLs)
D= diluted 1:10 with JAM cell line (EBV transformed HCV- PBLs)

FIG. 56

Alignment of all 9.2 sequences

```
H77C          CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT
9.2 final seq CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT
9.2a final seq CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT
9.2b final seq CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT
9.2c final seq CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT
9.2d final seq CACTCCCCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCT

H77C          AGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC
9.2 final seq AGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC
9.2a final seq AGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC
9.2b final seq AGCCATGGCGTTAGTATGAGTGTCGTACAGCCTCCAGGCCCCCC
9.2c final seq AGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC
9.2d final seq AGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCC

H77C          CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
9.2 final seq CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
9.2a final seq CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
9.2b final seq CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
9.2c final seq CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
9.2d final seq CCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACAC
```

- = clone alone (not diluted with other cells)
a= diluted 1:10 with MT4 cell line (HTLV1 transformed T cells)
b= diluted 1:10 with BJAB cell line (ATCC non-EBV transformed B cells)
c= diluted 1:10 with HLA 006 cell line (EBV transformed HCV- PBLs)
d= diluted 1:10 with JAM cell line (EBV transformed HCV- PBLs)
Red= Variation with respect to clone 9.2

FIG. 57A

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Alignment of all 9.2 sequences

H77C CGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATAAACCCGCT
9.2 final seq CGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATAAACCCGCT
9.2a final seq CGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATTAACCCGCT
9.2b final seq CGGAATTGCCGGAAGACTGGGTCCTTTCTTGGATAAACCCACT
9.2c final seq CGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATAAACCCGCT
9.2d final seq CGGAATTGCCAGGACGACCGGGTCCTTTCTTGGATTAATCCGCT

H77C CAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCG
9.2 final seq CAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCG
9.2a final seq CAATGCCTGGAGATTTGGGCGTGCCCCCGCGAGACTGCTAGCCG
9.2b final seq CTATGCCCGGCCATTTGGGCGTGCCCCCGCAAGACTGCTAGCCG
9.2c final seq CAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCG
9.2d final seq CAATGCCTGGAGATTTGGGCGTGCCCCCGCGAGACTGCTAGCCG

FIG. 57B

Alignment of all 9.2 sequences

```
H77C          AGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG
9.2 final seq AGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG
9.2a final seq AGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG
9.2b final seq AGTAGCGTTGGGTTGCGAAAGGCCTTGTGGTACTGCCTGATAGG
9.2c final seq AGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG
9.2d final seq AGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG

H77C          GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
9.2 final seq GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
9.2a final seq GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
9.2b final seq GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
9.2c final seq GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
9.2d final seq GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCA
```

FIG. 57B (Cont.)

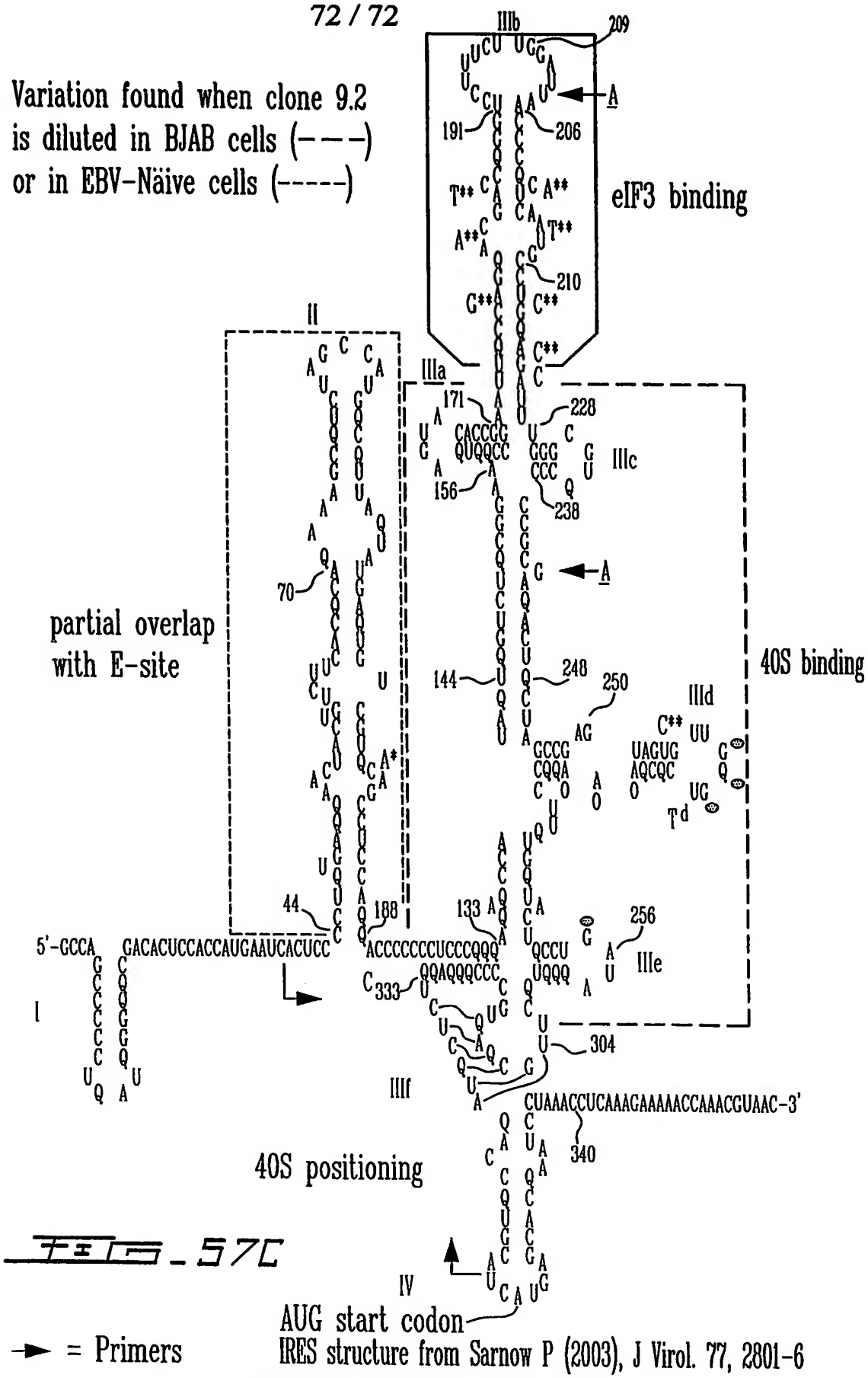
Variation found when clone 9.2
is diluted in BJAB cells (– – –)
or in EBV-Näive cells (-----)
IIIb
209
191
206
eIF3 binding
210
II
IIIa
171
228
IIIc
156
238
partial overlap
with E-site
70
40S binding
144
248
250
IIId
44
188
133
256
5'-GCCA GACACUCCACCAUGAAUCACUCC
ACCCCCCCUCCCQQQ
QQAQQQCCC
333
IIIe
I
304
IIIf
CUAAACCUCAAAGAAAAACCAAACGUAAC-3'
340
40S positioning
IV
AUG start codon
→ = Primers
IRES structure from Sarnow P (2003), J Virol. 77, 2801-6

FIG. 57C